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A Novel Parathyroid Protein in Chicken: Origin, Expression and Function

Pedro Luís Martins de Castro Pinheiro

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Orientador: Professor Doutor Adelino Vicente Mendonça Canário
Professor Catedrático
Universidade do Algarve

Co-orientador: Professor Doutor João José Oliveira Dias Coimbra
Professor Catedrático
Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

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será fruto da vossa educação.

“De raízes na terra e olhos no céu”

Luís de Castro Pinheiro

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List of publications, communications and sequences submissions

Articles

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Pinheiro, **P**.L., Cardoso J.C., Power D.M & Canario AVM (2011). "*Functional characterisation of the chicken PTH/PTHrP receptors: Evidence for loss of the PTH2R homologue in the avian lineage*." (*In preparation*).

Pinheiro, **P**.L., Cardoso J.C., Power D.M & Canario AVM (2011). "*A PTH-L gene in the elephant shark: a comment to Liu et al. 2010*". Journal of Bone and Mineral Research. (*In preparation*).

Pinheiro, **P**.L., Towers M., Bangs F., Tickle C., Power D.M & Canario AVM (2011). "*Abnormal bone development after chicken PTHrP and PTH-L knock-down"*. (*In preparation*).

Abstracts in Proceedings:

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Communications in meetings

Oral:

Pinheiro P, Cardoso J., Fuentes J., Power DM & Canário AVM (2008) "*A Novel Member and Comparative Analysis of the Parathyroid Hormone Family in Chicken***",** 24th Conference of European Comparative Endocrinologists (CECE), Genova, Italy.

Pinheiro P, Fuentes J., Power DM & Canário AVM (2009) *"The Parathormone and Related Peptides Activity and Expression in Chicken***",** 7º Congresso da Associação Ibérica de Endocrinologia Comparada (AIEC), Porto, Portugal.

Poster:

Pinheiro P, Cardoso J., Power DM & Canario AVM (2007) "*A novel Parathyroid Hormone Peptide in chicken: Bioinformatic and Gene Expression analysis*", 6th Conference of Iberian Association of Comparative Endocrinology (AIEC), Cadiz, Spain

Pinheiro P, Downie H., Fuentes J., Power DM & Canario AVM (2009) "*Functional Activity and Gene Expression of Parathormone*", XXXVIth International Union of Physiological Sciences (IUPS), Kyoto, Japan.

Pinheiro P., Downie H., Bangs F., Tickle C., Power DM & Canario AVM (2010) "*Parathyroid Hormone and Related Peptides during Chicken Development*", Second Annual Centre for Regenerative Medicine Symposium, Bath, UK.

Submissions to nucleotide sequence databases

- **FM955443** (PTH-L) *Gallus gallus* partial mRNA for parathyroid hormone-like peptide.

- **FR746109** (PTH1R) *Gallus gallus* partial mRNA for parathyroid hormone receptor 1.

- **FR746110** (PTH3R) *Gallus gallus* partial mRNA for parathyroid hormone receptor 3.

A novel parathyroid protein in chicken: origin, expression and function

Abstract

Calcium is a vital ion and the most abundant in vertebrates, involved in a myriad of functions. Calcium is tightly regulated by the endocrine system and its disruption has profound effects in the organism. In higher vertebrates, two peptide hormones, the parathyroid hormone (PTH) and PTH-related protein (PTHrP), are present from fish to mammals and are considered the principal hypercalcemic hormones. *PTH* and *PTHrP* shared a common ancestor and their emergence is suggested to be related with the acquisition of a bony skeleton early in vertebrate evolution. Both hormones possess a highly conserved N-terminal region (amino acids 1-34) which is essential for their calciotropic activity. Peptides of this family activate specific G-protein coupled receptors (GPCR) and in mammals two PTH receptors (*PTHR*) have been characterised, which, through several mechanisms, promote the increase of calcium concentration in plasma. A third receptor (*PTH3R*) has been proposed to be teleost fish specific. Also in fish, a third member of the PTH-family of peptides named PTH-Like peptide (*PTH-L)* was isolated and, despite scarce functional characterisation, it was found to stimulate calcium transport, although a specific receptor remains to be assigned. The presence of *PTH-L* in teleosts suggests the existence of additional functions associated with the vertebrate PTH-systems. The characterisation of the homologue system in other organisms such as amphibians and birds may contribute to elucidate about their functional evolution and mechanisms of regulation during the vertebrate lineage.

The overall aim of the present thesis was the functional characterization of the vertebrate PTH-system by investigating their origin, evolution and function in non-mammalian tetrapods. In this study, the chicken (*Gallus gallus*), a classical physiological model for calcium homeostasis with an intermediary position in the evolutionary scale between teleosts and mammals, was used to explore the evolution and function of the vertebrate PTH endocrine system, in particular of *PTH-L*. This was carried out through a) the isolation and characterization of the amphibian and chicken PTH-family members, b) the isolation and functional characterization of the chicken PTH receptors, c) the analysis of gene expression during chicken ontogeny and d) analysis of the action of PTH-family in chicken embryo skeletogenesis.

In chicken and amphibian, *PTH* and *PTHrP* genes with conserved homology for the mammalian and teleost homologues were identified. Moreover, *PTH-L* gene was found to be also present throughout the vertebrates with the exception of placental mammals. Splice variants of *PTHrP* and *PTH-L* are common in *Xenopus* and chicken and

the transcripts have a widespread tissue distribution being *PTHrP* the most expressed transcript while *PTH-L* expression is more restricted. *PTH* is widely expressed in fish tissue but from *Xenopus* to mammals it becomes largely restricted to the parathyroid glands (PTG). The N-terminal (1-34aa) region of PTH, PTHrP and PTH-L in *Xenopus* and chicken share significant sequence conservation and have the capacity to modify calcium fluxes across epithelia, suggesting a conserved role in calcium metabolism, possibly via similar receptors.

Two *PTHRs* were identified in chicken and they correspond to the homologues of the vertebrate *PTH1R* and teleost *PTH3R*, however the *PTH2R* gene remains to be identified in the bird lineage. The chicken receptors have a widespread expression starting from early stages of development and *PTH1R* is the most expressed transcript. The two receptors have affinity for the chicken (ck) PTH-family members (1-34aa N-terminal peptides) and are able to elicit cAMP production in a dose-dependent manner. While ckPTHrP highly stimulates cAMP in PTH1R, ckPTH-L is the less activating peptide for PTH3R and the ckPTH and ckPTHrP are the most potent peptides that stimulate PTH3R, approximately 7 times greater than PTH1R for the highest peptide concentration tested. ckPTHrP was the only peptide able to provoke PTH1R intracellular Ca^{2+} accumulation which is in agreement with its pleotropic functional role.

Ontogenic gene expression of the ckPTH-family members revealed that they are present in all the developmental stages analysed, being expressed since stage 4HH. The *PTHrP* is the most abundant transcript and RT-PCR detects several alternative splice isoforms with tissue specific expression supporting its paracrine profile. In contrast, the *PTH* and *PTH-L* are poorly expressed and their presence is restricted to certain tissues. *PTH-L* is expressed from at least 19 hours of incubation (stage 4HH) (using mix cDNA from whole body), and its expression was detected in stages related with central nervous system formation (stage 11HH), suggesting a different role from calcium homeostasis. *PTH* gene expression starts during the formation of the parathyroids (stage 24HH), and was strongly detected in the four parathyroid glands (PTGs) by *in situ* hybridization. However, it is also detected in limbs (stage 29HH) and may have different functions besides calciotropic activity.

Knock-down studies using morpholinos of the chicken *PTHrP* and *PTH-L* suggests an important role of the members of this family in the formation of the skeleton. PTHrP ablation during embryo wing development promotes the digit 3 bone development and decreases cartilage length. A similar observation was detected for PTH-L in which a decrease in cartilage length in the scapula was detected, however no alteration in bone were observed.

This work reports for the first time the chicken PTH endocrine system and highlights its conserved role in calcium transport and skeletal formation in non-mammalian tetrapods, among many other functions. The characterization of the tetrapod PTH-L and the absence of a *PTH2R* homologue in chicken raise novel questions about the evolution and function of the PTH-family members in vertebrates. It is hypothesized that they emerged prior to teleost divergence, via specie-specific gene duplication/gene deletion events, modulated by their living environment and physiological requirements in relation to calcium ion availability.

Resumo

O cálcio é um ião abundante e vital, presente em vertebrados, que está envolvido em várias funções. Este ião é minuciosamente regulado pelo sistema endócrino e uma desregulação da sua homeostasia pode resultar em efeitos profundos no organismo. A hormona da paratiroide (PTH) e o Péptido relacionado com a PTH (PTHrP) são duas hormonas pépticas presentes desde os peixes aos mamíferos, sendo as principais hormonas hipercalcémicas em vertebrados superiores. Estes factores endócrinos emergiram a partir de um gene ancestral comum e a sua origem está associada à aquisição de esqueleto ósseo no início da evolução dos vertebrados. A PTH e PTHrP partilham um elevado grau de conservação na região N-terminal (aminoácidos 1-34), que é essencial para a sua actividade calciotrópica. Os péptidos desta família activam receptores específicos acoplados à proteína G (GPCR). Em mamíferos dois receptores (PTHR) foram caracterizados, demonstrando-se que promovem o aumento da concentração de cálcio no plasma. Um terceiro receptor (PTH3R) foi identificado em peixes, onde recentemente também foi isolado um terceiro membro da família dos péptidos da PTH, designado por péptido semelhante à PTH (PTH-L). Apesar da sua pobre caracterização funcional, foi demonstrado que a PTH-L estimula igualmente o transporte de cálcio, contudo um receptor específico para este péptido permanece por descobrir. A presença da *PTH-L* em peixes sugere a existência de funções adicionais associadas ao sistema da PTH em vertebrados. A caracterização do sistema endócrino das hormonas e receptores desta família em outros organismos tal como anfíbios e aves, irá contribuir para uma melhor compreensão sobre a sua evolução e mecanismos de regulação em vertebrados.

O objectivo geral desta tese consistiu na caracterização do sistema das hormonas da família da PTH em vertebrados, investigando a sua origem, evolução e função em organismos tetrápodes não mamíferos. Neste estudo, a galinha (*Gallus gallus*), um

modelo clássico de estudos fisiológicos relacionados com a homeostase do cálcio e que possui uma posição intermédia na escala evolutiva entre os peixes e os mamíferos, foi utilizada para explorar a evolução e função do sistema endócrino das PTHs em vertebrados, em particular o da *PTH-L*. Este objectivo foi atingido através do a) isolamento e caracterização dos membros da família da PTH em anfíbios e galinha, b) isolamento e caracterização funcional dos receptores PTHRs, c) análise da expressão dos membros da famila das hormonas da PTH durante a ontogenia da galinha e d) análise da acção da família das PTHs na formação do esqueleto (esqueletogénese) em embriões de galinha.

Foram identificados em galinha e em anfíbios genes para a *PTH* e *PTHrP* com características semelhantes dos seus homólogos em mamíferos. O gene *PTH-L* foi identificado em todos os vertebrados com excepção dos mamíferos placentários. As variantes de splices da *PTHrP* e *PTH-L* são comuns em *Xenopus* e na galinha e os seus transcritos apresentam uma distribuição tecidular vasta, sendo a *PTHrP* o transcrito mais expresso e a *PTH-L* o mais restrito. A *PTH,* que apresenta uma distribuição ampla em vários tecidos em peixes, torna-se restrita à glândula paratiroide dos anfíbios aos mamíferos. A região N-terminal (1-34aa) da PTH, PTHrP e PTH-L em *Xenopus* e galinha possuem uma elevada conservação em termos de sequência e têm a capacidade de modificar os fluxos de cálcio em epitélios, indicando uma função conservada a nível do metabolismo do cálcio mediada possivelmente através de receptores semelhantes.

Dois *PTHRs* foram identificados em galinha e correspondem aos homólogos do *PTH1R* dos vertebrados e do *PTH3R* de teleósteos, porém o gene *PTH2R* continua por identificar em aves. Os receptores da galinha possuem uma expressão difundida desde as fases iniciais do desenvolvimento embrionário, sendo o *PTH1R* o transcrito mais expresso. Ambos os receptores são activados pelos membros da família PTH-(péptido 1-34aa N-terminal) de galinha (ck) através da estimulação da produção de AMPc intracelular de forma dose-dependente. O ckPTHrP é o péptido que mais estimula a produção de AMPc pelo PTH1R. O ckPTH-L é o menos estimulante para o PTH3R, sendo o ckPTH e o ckPTHrP os mais potentes, activando cerca de 7 vezes mais a produção de AMPc em comparação com o PTH1R para a maior concentração de péptido testada. O ckPTHrP foi o único péptido capaz de provocar a acumulação de Ca^{2+} intracelular o que está de acordo com o seu papel pleotrópico em vertebrados.

A expressão ontogénica dos membros da família da PTH demonstra que estão presentes em todas as fases do desenvolvimento analisadas e inicia-se no estadio 4HH. O transcrito *PTHrP* é o mais abundante, e as diversas isoformas de *PTHrP* possuem uma distribuição diferenciada por, RT-PCR, revelando um perfil parácrino. Em contrapartida, a *PTH-L* e a *PTH* são pouco expressas e sua presença é restrita a determinados tecidos. A

PTH-L foi detectada desde as 19 horas de incubação (4HH) (utilizando uma mistura de cDNA do embrião inteiro), e posteriormente em estádios relacionados com a formação do sistema nervoso central (11HH), sugerindo outra função além da homeostase do cálcio. A expressão génica da *PTH* inicia-se durante a formação das paratiroides (estadio 24HH) e através de hibridação *in situ* é muito abundante nas quatro glândulas paratiroides. No entanto, também foi detectada nos membros superiores do embrião (estadio 29HH), podendo ter diferentes funções para além da sua acção calcitrópica.

Estudos utilizando técnicas de *knock-down* com *morpholinos* específicos para a *PTHrP* e *PTH-L* de galinha, sugerem uma importante actividade destes genes na formação do esqueleto. A eliminação da tradução da *PTHrP* durante o desenvolvimento embrionário da asa promove o crescimento ósseo do dígito 3 e diminui o comprimento da cartilagem. Um resultado semelhante foi observado para a PTH-L, no qual ocorreu uma diminuição no comprimento da cartilagem da escápula, porém não foi registada nenhuma alteração a nível do osso.

Este trabalho descreve pela primeira vez o sistema endócrino da família da PTH em tetrápodes não mamíferos, destacando, entre outras possíveis funções, a sua actividade no transporte do cálcio e na formação do esqueleto. A caracterização da *PTH-L* em tetrápodes e a ausência de um gene homólogo para o *PTH2R* em aves originam novas questões sobre a evolução e a função dos membros desta família em vertebrados. É sugerido como hipótese que o sistema das PTHs e seus receptores emergiram antes da divergência dos peixes teleósteos por eventos de duplicação e eliminação de genes específicos em diferentes espécies. Esta evolução foi possivelmente condicionada pelas necessidades de adaptação fisiológica a diferentes ambientes, associadas à disponibilidade do ião cálcio.

CONTENTS

CHAPTER I

General Introduction

1. General Introduction

1.1. Overview

Calcium (Ca^{2+}) is a vital ion for survival and is involved in a wide range of physiological processes, from skeletal development, bone turnover or smooth muscle contraction to integrity and neural function. Sources of calcium depend on the environment and while terrestrial and freshwater vertebrates obtain it from their diet, seawater vertebrates obtain it from their natural environment (Bentley 1998).

The internal skeleton, a key feature of vertebrates, is an important pool for calcium and phosphate ions. This calcium can be mobilized from the skeleton when the extracellular calcium concentration is low and can be deposited when the extracellular calcium concentration is high (Sommerfeldt and Rubin 2001). However, from the total calcium that circulates in the blood stream, only a small proportion is tightly regulated by the endocrine system and still, an unbalance of the endocrine calcium homeostasis has profound effects in the organism (Bentley 1998; Sommerfeldt and Rubin 2001). The parathyroid hormone (PTH) and PTH-related protein (PTHrP) are the two principal calciotropic endocrine factors that play a fundamental role in body calcium homeostasis. In mammals, PTH is produced by the parathyroid glands (PTGs) and shares about 70% sequence and structural homology to PTHrP (Ingleton 2002; Potts 2005), acts as a hypercalcemic hormone (Munson 1955; Murray, Rao et al. 2005) and is the major hormone involved in calcium homeostasis (Potts 2005; Guerreiro, Renfro et al. 2007). Changes of Ca^{2+} concentration in circulation alter PTH secretion from the PTG via a negative feedback system. When Ca^{2+} concentration decreases, PTH secretion increases stimulating osteoclastic bone absorption, renal tubular calcium reabsorption and renal synthesis of 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] (Murray, Rao et al. 2005). These effects promote an increase in Ca^{2+} plasma concentrations. The biological actions of PTH occur when the N-terminal region of the molecule binds and activates specific receptors of family 2 G-protein coupled receptors (GPCRs) B1 (Gensure, Gardella et al. 2005), the parathyroid hormone receptor 1 (PTH1R) and 2 (PTH2R) (Juppner, Abou-Samra et al. 1991; Usdin, Gruber et al. 1995; Swarthout, D'Alonzo et al. 2002; Gensure, Gardella et al. 2005). On the other hand, PTHrP is an autocrine/paracrine factor produced in a wide variety of tissues with versatile and multifunctional effects, from adult to embryonic development (Clemens, Cormier et al. 2001). However, due to the high similarity and conservation of the N-terminal region for the mature peptide, PTH and PTHrP share the PTH1R, which assigns PTHrP calciotropic activity (Philbrick, Wysolmerski et al. 1996; Clemens, Cormier et al. 2001).

PTH and *PTHrP* origin is suggested to be related with the acquisition of a mineralised skeleton early in vertebrate evolution (Ingleton 2002; Potts 2005; Guerreiro, Renfro et al. 2007). These calciotropic hormones have been conserved throughout vertebrate evolution, teleost fishes possess duplicated genes for PTH and PTHrP, unlike mammals and birds which possess single copy genes (Ingleton 2002; Danks, Ho et al. 2003; Gensure, Ponugoti et al. 2004; Guerreiro, Renfro et al. 2007). Moreover, the N-terminal region is highly conserved among species, and the (1-34) amino acid region from the mature peptide was found to be sufficient for full biological activity (Gensure, Gardella et al. 2005). In addition to *PTH1R* and *PTH2R*, a third receptor, *PTH3R* is found (Rubin and Juppner 1999). *PTH3R* is closely related to *PTH1R*, is activated by both PTH and PTHrP, and was proposed to be a fish specific *PTH1R* duplication (Rubin and Juppner 1999; Gensure and Juppner 2005).

Recently a third related peptide designated PTH-Like peptide (PTH-L), with intermediate characteristics between PTH and PTHrP, was cloned from *Takifugu rubripes* (Canario, Rotllant et al. 2006). PTH-L also stimulates Ca^{2+} influx and the similarity of the N-terminal region to PTH and PTHrP led to the suggestion that it binds to the same receptors (Canario, Rotllant et al. 2006). The possibility that PTH-L has endocrine/paracrine function in fish suggests that determination of its localization, expression and activity in terrestrial vertebrates may help to clarify the evolution of this family of peptides and of the PTH gland in relation to calcium homeostasis. This is relevant because bioinformatics analysis has identified the *PTH-L* gene in the genome of a range of vertebrates from fish to marsupial mammals. However, it is not known if the *PTH-L* gene is expressed in those species, or if the functions are conserved. In this thesis it was decided to use chicken, *Gallus gallus*, an excellent developmental and physiological model (Brown, Hubbard et al. 2003; Davey and Tickle 2007) with growing genomic information available, to investigate the origin, expression and function of *PTH-L*.

1.2. Calcium homeostasis

Homeostasis is a physiological process defined as a condition of relative constancy, which is achieved through a variety of mechanisms that compensate for internal and external changes (Chiras 1999). Calcium has a tightly regulated homeostasis and is the most universal carrier of biological signals involved in cell life, from its origin at fertilization to its end in the apoptotic processes, by playing a fundamental role in a number of important physiological processes. Cells need Ca^{2+} to correctly carry out most of their vital functions (Krebs and Michalak 2007), such as bone formation and maintenance, nerve depolarization, smooth muscle contraction, integrity and neuronal function, intracellular signalling and also in all processes that involve exocytosis including hormone release and action (Bentley 1998). In vertebrates, calcium is absorbed mainly in the duodenum and upper jejunum into the vascular system and stored almost entirely as hydroxyapatite crystal of calcium phosphate (Johnston and Ivey 2002). The circulating Ca^{2+} levels vary considerably among vertebrates and during the different stages of their life cycle. In adult humans, normal total calcium level are from 2.2 to 2.6 mM (Boron and Boulpaep 2009) which in disease situations may reach 3.5 mM (Starker, Bjorklund et al. 2010). In birds, the extracellular calcium pool contains 2.2 to 3 mM (Vitti and Kebreab 2010) and these values duplicate in egg-laying hens to reach 5 to 7.5 mM (Etches 1987) in order to promote Ca^{2+} flow to the egg construction in the egg shell gland (ESG) (Bar 2009).

During vertebrate evolution, a feedback mechanism which detects changes in plasma Ca^{2+}, by Ca^{2+}-sensing receptor (CaSR) (Brown, Gamba et al. 1993), was developed and refined (Bar 2008). This mechanism aimed at the maintenance of extracellular calcium concentration within physiological levels and involves the intestine, kidney and bone under the regulation of PTH (Potts 2005; Guerreiro, Renfro et al. 2007), calcitonin (Findlay and Sexton 2004) and the 1,25-dihydroxycholecalciferol or vitamin D (Dusso, Brown et al. 2005). However, other hormones participate in this complex process, such sex steroid hormones, glucocorticoids, growth hormone and prolactin (Bentley 1998; Bar 2008; Boron and Boulpaep 2009). In mammals, PTH is secreted in response to a lowering of blood calcium levels and modulates the activity of specific bone and kidney cells by raising calcium levels back to their normal physiological concentration (Potts 2005). In contrast, calcitonin has a counteracting action to PTH and is released in response to elevated blood calcium levels by inhibiting osteoclastic bone resorption (Mundy and Guise 1999). The vitamin D precursor is either ingested by the diet or synthesized by the skin after exposure to ultraviolet sun light and take the active form 1,25-dihydroxy vitamin D_3 [$1,25(OH)_2D_3$] in kidney, where is produced in response to

phosphate, calcium and PTH (Dusso, Brown et al. 2005). 1,25-dihydroxy vitamin D_3 increases calcium/phosphate concentration in plasma by increasing their absorption in the gastrointestinal tract, increasing bone resorption and enhancing the effects of PTH in renal tubular calcium reabsorption (Mundy and Guise 1999; Dusso, Brown et al. 2005; Bar 2008) (Figure 1).

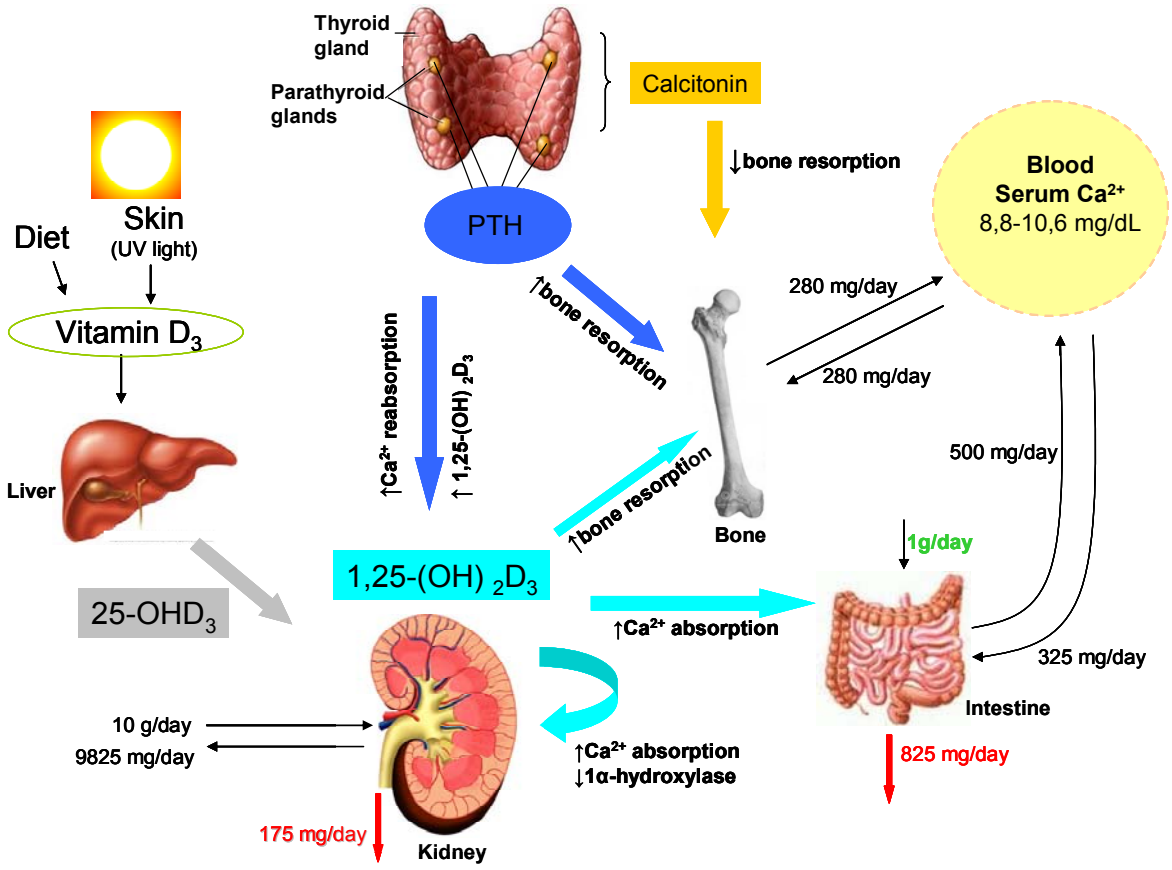

Figure 1: Scheme of the calcium balance and related hormones involved in its regulation in human. The hormone actions are represented by different colored arrows for each hormone, calcium flux by black arrows and red arrows represent calcium excretion.

1.3. Calcium transport

Calcium transport may occur both through the transcellular *(through the cell)* and the paracellular *(between cells)* way. In the transcellular transport four protein groups appear to be involved, the calbindins, epithelial calcium channels (transient receptor potential vanilloid - TRPVs), plasma membrane calcium-ATPase (Ca^{2+}ATPase or PMCA) and sodium-calcium (Na^{+}/Ca^{2+}) exchangers (NCX). Calcium uptake requires the epithelial calcium channel TRPV5 and TRPV6, calbindin transports calcium across the cell and the

plasma membrane calcium-ATPase and NCX mediate the final delivery of calcium to the bloodstream (Hoenderop, Nilius et al. 2003) (Figure 2). Paracellular transport is believed to include tight junction proteins (Bar 2009).

Calbindins are proteins considered to facilitate Ca^{2+} movement in the epithelial cells of calcium-transport organs, associated in the protection of cells from high concentrations of Ca^{2+} or from apoptotic cellular degradation (Hoenderop, Nilius et al. 2003; Bar 2008). Calbindins were found to be highly expressed in classical calcium massive transport tissues such as kidney, intestine, placenta and uterus. However they are also expressed in tissues related to calcium homeostasis like bone, tooth, parathyroid cells, and in tissues not directly related to calcium homeostasis such as nervous system, pituitary, pancreas and testes, where they are also regulated by estrogens (Choi, Leung et al. 2005; Nguyen, Lee et al. 2005).

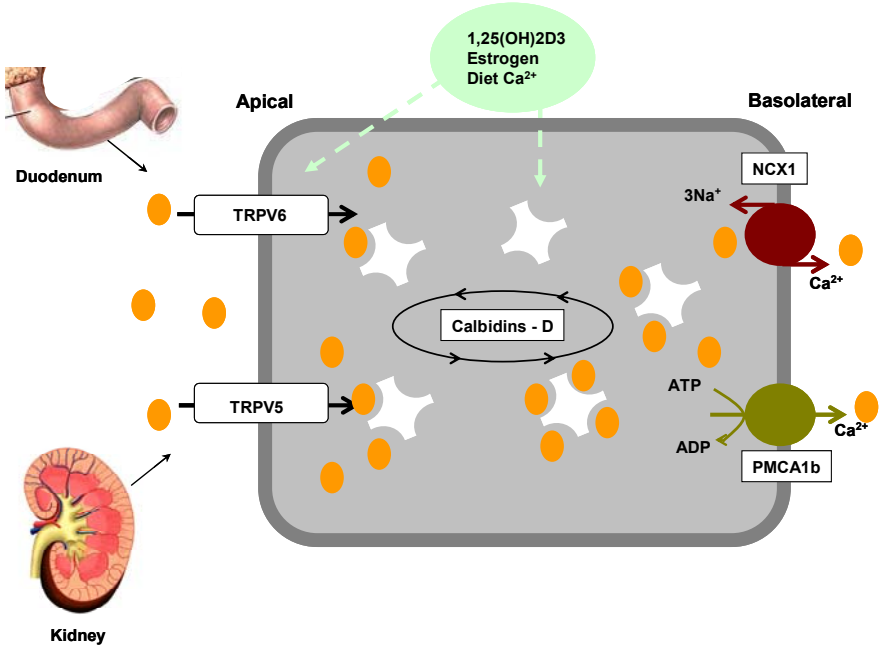

Figure 2: The three-step-process of transcellular Ca^{2+} transport in the kidney and intestine (Hoenderop, Nilius et al. 2003). Entry of Ca^{2+} through theTRPV5 and TRPV6, in cytosol is buffered by the calbindins and at the basolateral membrane, Ca^{2+} is extruded via PMCA1b and NCX1.

The transient receptor potential (TRP) is a super family of channels involved in ion transport into the cells. It comprises 6 subfamilies in which TRPV5 and TRPV6 are considered to facilitate calcium entry into epithelial cells of calcium organs, regulated by $1,25(OH)_2D_3$, estrogens and Ca^{2+} in the mammalian upper intestine, distal nephron, bone, placenta and uterus, with TRPV5 much more highly expressed in kidney and TRPV6 in duodenum (Venkatachalam and Montell 2007). The PMCAs need ATP to transport Ca^{2+} out of the cells against an electrochemical gradient, frequently located in the epithelial

basolateral side of intestine, kidney and placenta. However, in some organisms, PMCAs expresses in the apical membrane of the tubular gland cells, such egg shell gland, and unlike intestinal and renal, are not modulated by $1,25(OH_2)D_3$. The Na^+/Ca^{2+} exchangers (NCX) are transporter proteins encoded by at least three genes (Lytton 2007) being the NCX1 the most widely expressed in the calcium classical transport tissues (intestine and kidney) but also found in osteoblast cells *(cell responsible for bone formation)* (Stains, Weber et al. 2002; Hoenderop and Bindels 2005; Hoenderop, Nilius et al. 2005). NCX1 expression, at least in kidney, is regulated by PTH and $1,25(OH)_2D_3$ in contrast to intestine where activity is not affected by $1,25(OH)_2D_3$ (Bar 2009).

1.4. Endocrine regulation of calcium

In adult humans, from approximately 1000mg of dietary calcium intake in a day, ~175mg is absorbed intestinally, the same amount as urinary excretion. Bone absorption and formation correspond to 280mg of calcium turnover (Figure 1). These exchanges maintain a free ionized Ca^{2+} plasma concentration tightly regulated between 1 and 1.3 mM, what corresponds to ~45% of circulating calcium; ~45% is bound to proteins (mainly albumin) and only 10% is complexed with low molecular weight organic anions (e.g. citrate and oxalate) (Mundy and Guise 1999; Boron and Boulpaep 2009).

Calcium homeostasis is closely related to phosphate balance, the two are the principal components of hidroxyapatite crystals which correspond to the mineral phase of the vertebrate bone. The regulation of both ions is mainly dependent on the action of three hormones: PTH, vitamin D ($1,25(OH)_2D_3$) and calcitonin that act on three target organs: bone, gut and kidney (Mundy and Guise 1999) (Figure 1).

1.4.1. Vitamin D

In the mammalian body, vitamin D exists in two forms, vitamin D_3 (cholecalciferol) and vitamin D_2 (ergocalciferol). These two molecules differ in the side chain of ring D, vitamin D_3 is a derivative of cholesterol in contrast to vitamin D_2 which is derived from the plant sterol ergosterol (Dusso, Brown et al. 2005). The steroid hormone $1,25(OH)_2D_3$ is the major biologically active metabolite of vitamin D_3, and may be ingested in the diet or synthesized in the skin from 7-dehydrocholesterol through photolytic conversion from exposure to UV radiation from sunlight (Figure 3). Vitamin D_2 can only be obtained from the diet (Webb and Holick 1988; Dusso, Brown et al. 2005; Boron and Boulpaep 2009).

The best known functions of $1,25(OH)_2D_3$ are to promote the elevation of serum calcium and phosphate by increasing calcium and phosphate absorption from the gastrointestinal tract, and to enhance the effects of PTH on the nephron to promote renal tubular calcium reabsorption and phosphate loss (Dusso, Brown et al. 2005). It also stimulates differentiation of osteoclast *(bone cell with function in resorption and degradation of existing bone)* precursors, causing their maturation and leading to bone resorption (Suda, Takahashi et al. 1992).

In humans, vitamin D hydroxylation occurs in the liver to form 25-hydroxyvitamin D (by 25-hydroxylase), the substrate for hydroxylation in the proximal nephron of the kidney by 1α-hydroxylase, to form $1,25(OH)_2D_3$ (Figure 3). Dietary calcium can regulate enzyme activity directly through changes in serum calcium and indirectly by altering PTH levels (Dusso, Brown et al. 2005), however, $1,25(OH)_2D_3$ also acts via its receptor to inhibit renal 1α-hydroxylase activity (Mundy and Guise 1999). Dietary phosphate restriction also increases renal 1α-hydroxylase activity independently of changes in PTH and calcium (Dusso, Brown et al. 2005). After secretion, $1,25(OH)_2D_3$ has a half-life ~5 hours in humans, with half percent excreted as urinary metabolites and the rest as fecal metabolites.

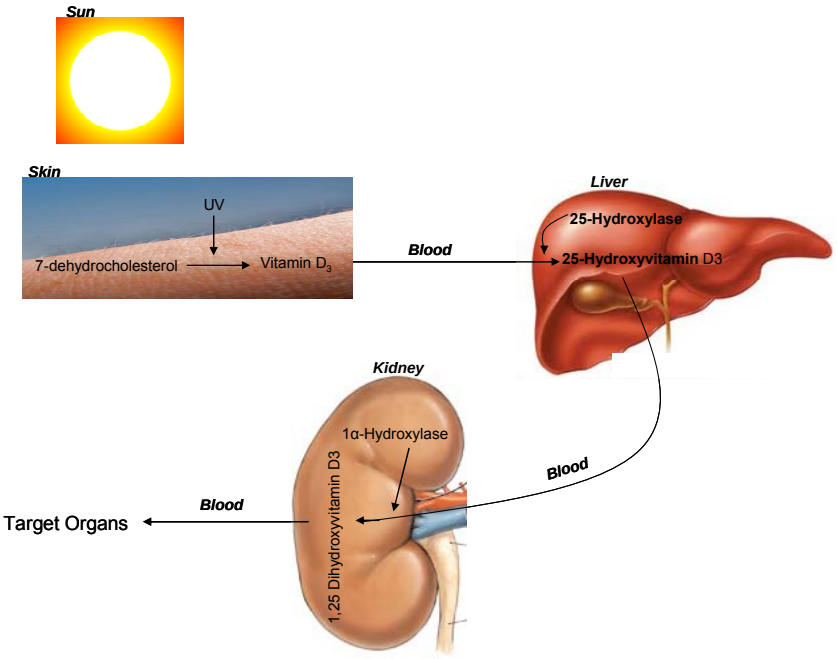

Figure 3: Synthesis of 1,25-dihydroxyvitamin D_3 after sun light exposure [adapted from (Fox 2002)].

The responses of vitamin D are too rapid to involve changes in gene expression and most of the biological activities of $1,25(OH)_2D_3$ require a high-affinity receptor, the Vitamin D Receptor (VDR). In the small intestine, calcium uptake is vitamin D dependent, the TRPV5 and TRPV6 channels are regulated by $1,25(OH)_2D_3$ and studies using VDR-

knockout mice revealed a reduced TRPV channels expression. Moreover, $1,25(OH)_2D_3$ also stimulates the expression of the Na-Pi co-transporter increasing phosphate uptake. In the skeleton, it has been reported that optimal osteoblastic bone formation and osteoclastic bone resorption demand both $1,25(OH)_2D_3$ and the VDR (Panda, Miao et al. 2004). $1,25(OH)_2D_3$, as well as PTH and prostaglandins, stimulate RANKL expression which binds to RANK inducing a signalling that results in the differentiation and maturation of osteclasts (discussed later) (Kitazawa, Kajimoto et al. 2003). Experiments using $1,25(OH)_2D_3$-VDR-defective mutants mice showed an increase in osteoblast number, serum alkaline phosphatase, bone formation, and bone volume (Panda, Miao et al. 2004). These findings suggest that the $1,25(OH)_2D_3$/VDR system may have an effect necessary to induce bone turnover. Moreover, in kidney, $1,25(OH)_2D_3$ controls its own homeostasis through the suppression of 1α-hydroxylase. However, it also enhances renal calcium reabsorption, by regulating calbindin and TRPV5 expression and accelerating PTH dependent calcium transport in the distal tubule (Bar 2008). Apart from these functions, vitamin D has several non-classical actions in different tissues, such as suppression of cell growth, regulation of apoptosis, control of skin differentiation, control of insulin secretion, control of muscle function, control of the nervous system, which could suggest the involvement of a different type of receptors (Bikle 2009).

1.4.2. Calcitonin

In mammals, calcitonin is a 32 amino acid peptide synthesised in a number of tissues, especially the C cells (or parafollicular cells) of the thyroid gland (Findlay and Sexton 2004). Alternative splicing of the calcitonin gene give rise to different active peptides. In the C cells calcitonin is the significant transcript, however in the brain, a different splice form gives rise to the calcitonin gene-related peptide (CGRP) which acts as a neurotransmitter (Findlay and Sexton 2004; Boron and Boulpaep 2009). CT has the ability to lower the concentration of plasma Ca^{2+} due to an inhibitory action on osteoclast-mediated bone resorption and is regulated by serum calcium levels and gastrointestinal peptide hormones (Mundy and Guise 1999; Findlay and Sexton 2004; Hong, Choi et al. 2007; Boron and Boulpaep 2009)

The osteoclasts, which lack a PTH receptor, possess the receptor for CT, a GPCR family member which activates either the adenylyl cyclise or phospholipase C. CT receptor is widely expressed, however, the role of CT in calcium homeostasis has been hard to define. In adult humans, large doses of CT have little effect on serum calcium levels (Boron and Boulpaep 2009) and neither CT deficient patients nor patients with CT

excess have alterations in calcium homeostasis, maintaining the plasma levels of Ca^{2+}, vitamin D and PTH, what suggests a tiny role in calcium balance (Mundy and Guise 1999; Findlay and Sexton 2004; Hong, Choi et al. 2007). In contrast, in teleost fishes, CT is expressed in the ultimobranchial gland, inhibiting osteoclast resorption which leads to osteoclast cell membrane contraction decreasing bone resorption, and causing Ca^{2+} to return to normal concentrations (Mundy and Guise 1999). The salmon CT differs in 14 amino acids from the human hormone and is 10 fold more potent than the latter in inhibiting osteoclast function (Boron and Boulpaep 2009). Interestingly, treatment with parathyroid hormone in thyro/parathyroidectomised rats (without CT) caused more bone loss than in intact rats (Yamamoto, Seedor et al. 1995). However, after hours of exposure of osteoclasts to high calcitonin concentrations the antiresorptive action begins to fall. These results have limited the use of calcitonin in the clinical treatment of hypercalcemic diseases (Boron and Boulpaep 2009).

1.4.3. Parathyroid hormone family members

In vertebrates, the members of the PTH-family share a role in calcium transport and counteract calcitonin action. In humans, when there is a slight increase in circulating Ca^{2+} concentration (from 1.1 to 1.3 mM) the plasma levels of PTH reduce drastically (from 75 to 15 ng/l) and PTH release can fall from 100% to nearly 0% when Ca^{2+} concentration increases from 1 to 1.5 mM, which emphasizes the very active role of this hormone in maintenance of constant calcium levels (Guerreiro, Renfro et al. 2007). The levels of Ca^{2+} in the blood are detected by the CaSR, a member of G-coupled receptor family, which is localized in the PTGs and respond to the increase of Ca^{2+} by inhibiting PTH production (Brown, Gamba et al. 1993; Bentley 1998; Ariyan and Sosa 2004) (Figure 4). The mechanism involved in the inhibition of PTH synthesis involves the activation of intracellular messengers (detail on GPCRs and signalling pathways will be given when PTHRs are discussed). CaSR activates the intracellular secondary messenger phospholipase C, generating inositol 1,4,5-triphosphate (IP3) and diacyglycerol (DAG) resulting in intracellular calcium release and protein kinase C (PKC) activation which inhibits PTH synthesis and release (Boron and Boulpaep 2009) (Figure 4).

PTH is the most studied member of the parathyroid hormone family. It is a classical endocrine factor, produced by a gland, while the other two members have a widespread tissue distribution and paracrine functional roles, some of which remain to be studied. The high homology in N-terminal (1-34) amino acid region of the mature peptide of the PTH members allow PTH and PTHrP to share the same receptor type (PTH1R) and

calciotropic effects (Gensure, Gardella et al. 2005).The first 13 amino acids of the mature precursor peptide are the most conserved residues within the family members across vertebrates and are essential for receptor activation (Gensure, Gardella et al. 2005).

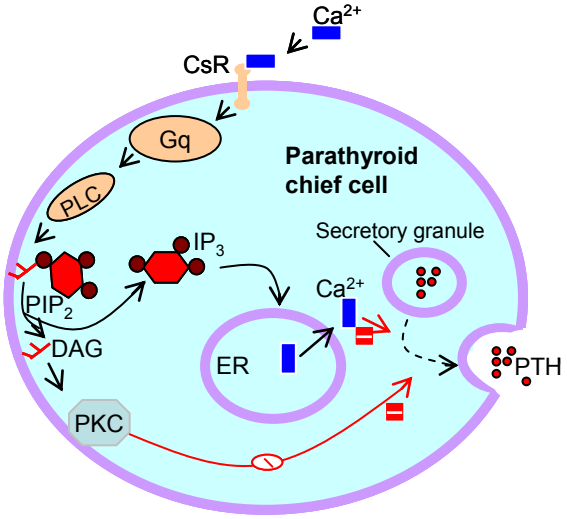

Figure 4: PTH secretion and CasR regulation in the parathyroid chief cells. Calcium binds the receptor coupled to G protein activating phospholipase C which converts phosphoinositides (PIP2) to IP3 and DAG. IP3 causes internal calcium release and DAG stimulates PKC. IP3 and PKC inhibit PTH release and the increase of intracellular calcium also inhibits PTH synthesis (adapted from (Boron and Boulpaep 2009)). ER: endoplasmatic reticulum.

1.4.3.1. Parathyroid hormone (PTH)

In mammals, PTH is produced by the chief cells of the PTGs, sharing about 70% sequence and structural homology with PTHrP (Ingleton 2002; Potts 2005). In humans, a precursor molecule pre-pro-PTH, containing 115 amino acids, undergoes cleavage at two sites giving rise to a pro-PTH with 90 amino acids, which in the Golgi apparatus becomes the mature biologically active 84-amino acid peptide composed of the 1-34 N-terminal fragment (biologically active) and the C-terminal fragment (Habener 1976) (Figure 5).

PTH is secreted mainly in response to a decrease in ionized serum calcium levels, acting as a hypercalcemic hormone (stimulates the increase of calcium in plasma). However, a decrease of vitamin D and an increase of plasma phosphorus concentration also stimulate PTH release (Mundy and Guise 1999; Potts 2005; Boron and Boulpaep 2009). Parathyroid cells exposed *in vitro* to hypercalcemic conditions (calcium plasma concentration elevated to reference values) display a decrease in mRNA for *PTH* while those exposed to hypocalcemic conditions (calcium plasma concentration below reference

values) did not show an increase which suggests *PTH* synthesis is likely maximal under normal physiological conditions (Russell, Lettieri et al. 1983).

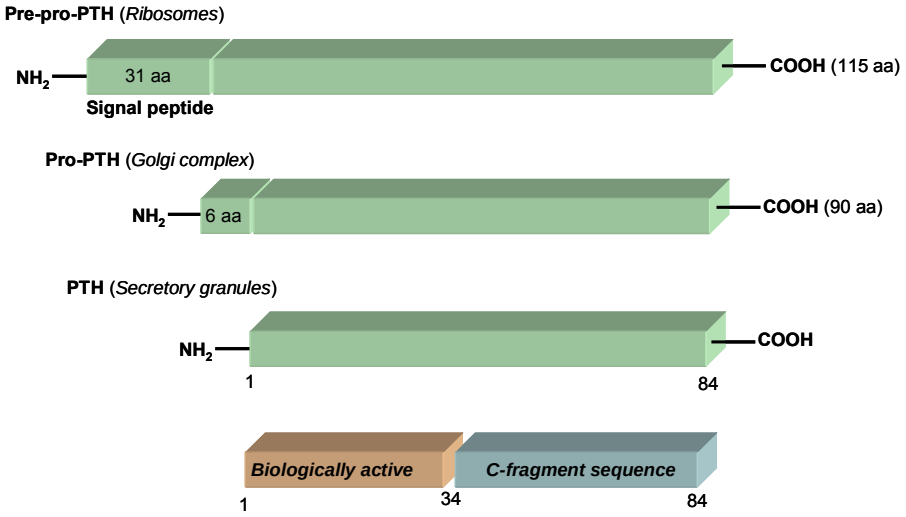

Figure 5: Schematic representation of the human PTH precursor structure and modifications during synthesis and after release by the PTG (Boron and Boulpaep 2009).

Once secreted, PTH circulates in the blood and the intact biological active peptide has a half-life in circulation of about 4 min. Intact PTH is cleaved by the liver, in N-terminal fragments and a larger C-terminal fragment (Mundy and Guise 1999). The biological activity of PTH is conferred by the N-terminal region, which is hydrolyzed in kidney. However the C-terminal region may have discrete biologic properties with a half-life much longer than N-terminal or intact PTH because is cleared exclusively by glomerular filtration (Mundy and Guise 1999; Cranney and Papaioannou 2006; Boron and Boulpaep 2009). PTH secretion stimulates osteoclastic bone absorption, renal tubular calcium reabsorption and, along with prolactin, growth hormone (GH) and 17β-oestradiol, renal synthesis of 1,25-dihydroxyvitamin D_3 (Barnicot 1948; Garabedian, Holick et al. 1972; Swarthout, D'Alonzo et al. 2002; Murray, Rao et al. 2005; Canario, Rotllant et al. 2006) which in turn increases intestinal calcium and phosphate absorption (Munson 1960; Murray, Rao et al. 2005).

In the kidney, there is a large decrease of Ca^{2+} excretion when PTH stimulates distal Ca^{2+} reabsorption, raising significantly its plasma concentration. At the same time, PTH reduces PO_4^{3-} reabsorption in proximal and distal tubules, promoting phosphaturia, increasing PO_4^{3-} elimination. This is essential, because if PO_4^{3-} concentration increased along with Ca^{2+} it could cause $CaPO_4$ salts precipitation, inhibiting the rise in plasma Ca^{2+} concentration by the action of PTH (Guerreiro, Renfro et al. 2007; Boron and Boulpaep 2009).

PTH secretion stimulates osteoclastic bone absorption, however, intermittent doses of PTH (1-34 aa), used as osteoporosis treatment, have bone synthesis effects (Tam, Heersche et al. 1982; Neer, Arnaud et al. 2001). Osteoclastic cell proliferation is indirectly regulated by the PTH which binds to osteoblast. The osteoblast expresses two molecules essential for osteoclastogenesis, the macrophage colony-stimulating factor (M-CSF) and the receptor for activation of nuclear factor kappa B (NF-Kappa B) (RANK) ligand (RANKL) (Teitelbaum 2000; Schlesinger and Thiele 2010). PTH binds the PTH receptor stimulating osteoblastic cells to secrete these factors, causing the osteoclast proliferation leading to bone resorption and inhibit the expression of osteoprotegerin (OPG) (a “decoy” receptor for RANKL) defining its catabolic role in bone. Indeed, it is the balance between the expression of the stimulator of osteoclastogenesis, RANKL, and of the inhibitor, OPG, that dictates the quantity of bone resorbed (Teitelbaum 2000; Khosla 2001).

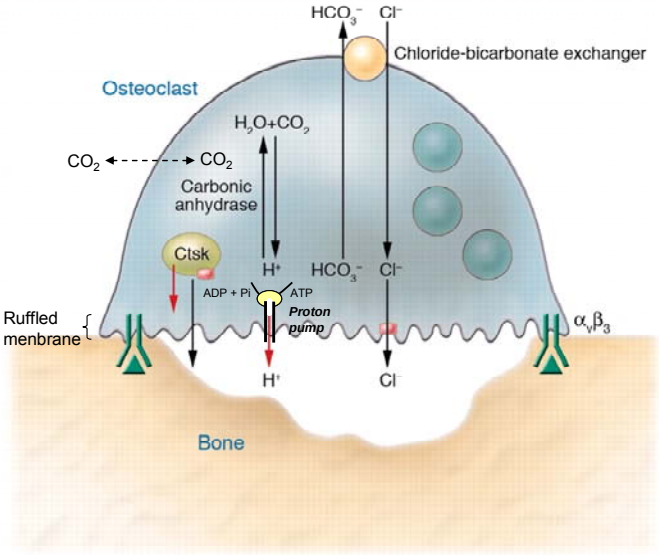

Figure 6: Mechanism of osteoclastic bone resorption. RGD is represented by green triangle and cathepsin K (Ctsk) [adapted from (Mozo and Anghel 2006)].

Bone resorption is a process initiated by the proliferation of immature osteoclast precursors, which are multinucleated cells from hematopoietic origin (formed by the fusion of mononuclear progenitors of the monocyte/macrophage family) (Coccia, Krivit et al. 1980; Lacey, Timms et al. 1998), which acquire osteoclast phenotype and finally, degrade the organic and inorganic phases of bone. The osteoclast fixes on bone, via binding of arginine–glycine–aspartic acid (RGD)-containing proteins to the integrin $\alpha_v ß_3$, forming a ruffled membrane (Teitelbaum 2000). The degrative process is initiated by hydration of carbon dioxide to carbonic acid, which dissociates into protons and bicarbonate ions. These protons are transited into the isolated microenvironment and, in antiresorptive

plasma membrane, HCO_3^- is exchanged for Cl^-, which passes the osteoclast and is secreted into the space between itself and bone. The high acidic environment promotes the bone mineral mobilization and exposes the organic matrix of bone (type I collagen and non-collagenous proteins) which is degraded by cathepsin and in the end, solubilised mineral components are released when the cell migrates (Figure 6) (Teitelbaum 2000; Teitelbaum 2000).

In a different scenario, PTH can also promote bone mineralization by two mechanisms: a) PTH can promote bone synthesis directly by activating Ca^{2+} channels from bone fluid to the osteocyte which transfer Ca^{2+} to the osteoblasts in the bone surface or b) it can also promote bone synthesis indirectly, in that osteoclastic bone reasorption leads to the release of growth factors such as IGF-1 and 2 and transforming growth factor β (Boron and Boulpaep 2009) which promote the bone formation and mineralization (Ishibe, Ishibashi et al. 1998; Iwata, Hosokawa et al. 2010)

1.4.3.2. Parathyroid hormone-related Protein (PTHrP)

PTHrP was first discovery in 1987 and was characterised as a circulating peptide responsible for the elevated levels of Ca^{2+} in human blood, associated with the syndrome of humoral hypercalcemia of malignancy (HHM) (Moseley, Kubota et al. 1987; Abbink and Flik 2006). Similar to hyperparathyroidism, uncontrolled PTHrP secretion produces Ca^{2+} reabsorption from bone and suppress urinary Ca^{2+} loss (Philbrick, Wysolmerski et al. 1996; Guerreiro, Renfro et al. 2007). PTHrP is produced in a wide variety of tissues and acts locally. Unlike PTH, a specific site for the production of PTHrP has not been identified and expression studies carried out in vertebrates revealed that this hormone has a vast distribution, which seems to act has an endocrine/paracrine factor with multiple functional roles associated (Guerreiro, Renfro et al. 2007).

In mammals, PTHrP is synthesized with 36 amino acids in the pre-pro region and is cleaved to produce three different transcript isoforms (of 139, 141 and 173 amino acids) by differential alternative splicing. The PTHrP isoforms undergo post-translational proteolytic cleavage originating three different regions, N-terminal (1-36), mid region (38-94) and C-terminal (107-139) PTHrP peptides (Figure 7) (Philbrick, Wysolmerski et al. 1996; Clemens, Cormier et al. 2001; Ingleton 2002). Each peptide fragment acts through distinct receptors and has its own biological properties thus explaining the multiple activities detected in mammals (Clemens, Cormier et al. 2001). For example in bone PTHrP (1-36) stimulates bone resorption, while PTHrP (107-139) inhibits bone resorption (Philbrick, Wysolmerski et al. 1996).

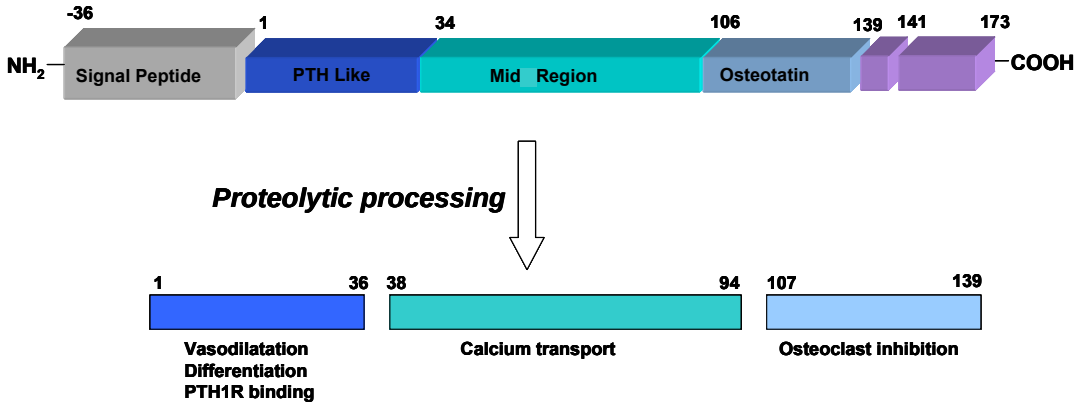

Figure 7: Human PTHrP precursor structure and mature peptide with biological function after posttranslational processing (Clemens, Cormier et al. 2001).

The first 13 amino acids of the precursor within the 1-34 N-terminal region, exhibit high homology to PTH but the remaining peptide segments are divergent. Like PTH, PTHrP bioactivity requires the presence of this intact amino terminal region, because removing the first two amino acids of N-terminal region reduces dramatically the capacity to bind to the receptor (Rabbani, Mitchell et al. 1988; Rotllant, Guerreiro et al. 2006). The N-terminal domain of PTHrP stimulates protein kinase A (PKA), protein kinase C (PKC) and/or calcium dependent pathways when it activates PTH1R (Mannstadt, Jüppner et al. 1999). The PTHrP mid-region, which contains a bipartite nuclear localization sequence (nuclear localization signal, at residues 88-91 and 102-106) and an importin β *(sub unit of importin, a protein which moves other proteins into the nucleus)* binding site (at 66-94), was recently suggest to be responsible for the functions of PTHrP on morphogenesis, cell proliferation, apoptosis, and calcium homeostasis instead of N-terminal region (Toribio, Brown et al. 2010). However, no receptor has been identified to this region. The C-terminal domain (osteostatin), is a potent osteoclastic bone resorption inhibitor, and it was suggested that the growth inhibition and the C-terminal specific signalling occur through a different receptor from PTH1R (Cuthbertson, Kemp et al. 1999).

In tetrapods, PTHrP is a versatile factor with multifunctional effects, from embryonic and foetal development to adult (Chan, Strewler et al. 1990; Ingleton 2002; Naveh-Many 2005; Abbink and Flik 2006). PTHrP physiological functions are mainly related to transepithelial calcium transfer (renal, placental, oviduct, mammary gland), smooth muscle regulation (vascular, intestinal, uterine, bladder) and control of skeletal development, cell growth development and differentiation of tissues (Philbrick 1998; Lanske, Amling et al. 1999; Clemens, Cormier et al. 2001). Studies of gene knock-out mice established that skeletal development and growth require PTHrP (Philbrick, Wysolmerski et al. 1996) and potential local effects in bone and cartilage differentiation

have also been reported for PTHrP, where it plays an important chondrocyte differentiation inhibition, establishing a feedback to Indian Hedgehog gene (IHH) (Minina, Kreschel et al. 2002).

PTHrP is essential for survival and development and the identification of new features in different organisms may help to clarify its roles and understand its calcemic effects.

1.4.3.3. The Parathyroid Hormone receptors (PTHRs)

The described PTH biological actions occur when the amino terminal end of the PTH molecule binds and activates specific receptors of the family 2 G-Protein Coupled Receptors (GPCRs) B1 (Gensure, Gardella et al. 2005). The parathyroid hormone receptor 1 (PTH1R), expressed mainly in bone cells and kidney (Juppner, Abou-Samra et al. 1991; Swarthout, D'Alonzo et al. 2002), however with a widespread expression in a range of tissues (Usdin, Gruber et al. 1995), and PTH2R which is present mainly in the hypothalamus-brain region (Usdin, Gruber et al. 1995). PTH and PTHrP share the same PTH1R and calciotropic activity through the highly conserved 1-34 N-terminal region of mature peptide. Mammalian PTH binds the PTH1R and PTH2R while PTHrP only binds to PTH1R (Gensure, Gardella et al. 2005). However, PTH2R is also activated by a structurally unrelated neuropeptide, the tuberoinfudibular peptide 39 (TIP39) and activates different types of response which remain to be clarified (Papasani, Gensure et al. 2004).

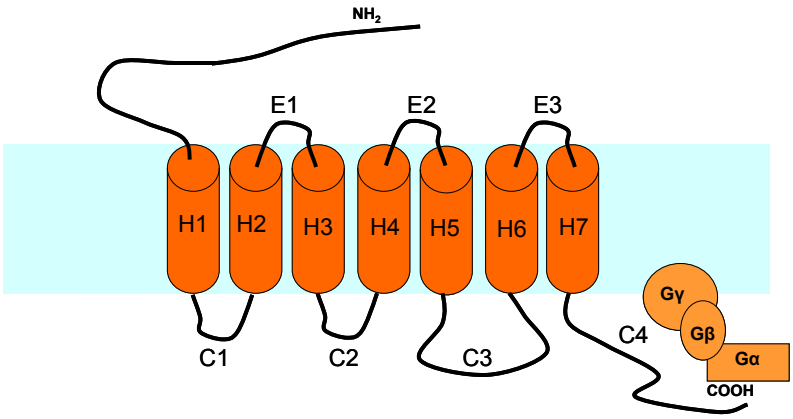

Figure 8: Schematic representation of a G-protein coupled receptors (GPCRs) and the G-proteins (α, β and γ). Transmembranar regions represented with “H”, extracellular loop with “E” and intracellular with “C”.

GPCRs are characterised by the presence of highly conserved seven putative transmembrane segments and a long N-terminal region (see Figure 8), which contain six

conserved cysteines residues involved in the formation of the binding pocket and in ligand-binding interaction (Harmar 2001; Cardoso, Pinto et al. 2006). GPCRs activation in the presence of their cognate ligand induce conformational changes in the transmembrane domains (Gether 2000) and in the cytosol, the C-terminal region trigger the intracellular signalling mechanisms that interact with G proteins (heterotrimeric GTPases) to regulate the synthesis of intracellular second messengers such as cyclic AMP, inositol phosphates, diacylglycerol and calcium ions (Harmar 2001).

1.4.3.4. PTH-family members in non-mammalians

PTH and *PTHrP* gene homologues have also been described in non-mammalian vertebrates (Guerreiro, Renfro et al. 2007), and one of the most recent additions to this family, designated PTH-Like peptide (*PTH-L*), was cloned from the teleost fish *Takifugu rubripes* (Canario, Rotllant et al. 2006). *PTH-L* shares intermediate structural and putative functional characteristics with PTH and PTHrP and high sequence similarity in the 1-34 N-terminal amino acid region as well to PTH and PTHrP, suggesting it may bind to the same receptors (Canario, Rotllant et al. 2006). Physiological assays performed in fish revealed it is also able to promote Ca^{2+} influx. However, it is not known if the PTH-L features described in teleosts are conserved across the vertebrates and if the protein product of this gene has other functions, if it is an endocrine or paracrine factor or even where it is produced. Determination of PTH-L localization and function in terrestrial vertebrates may help to clarify the evolution of PTH-family system and the mammalian calcium homeostasis.

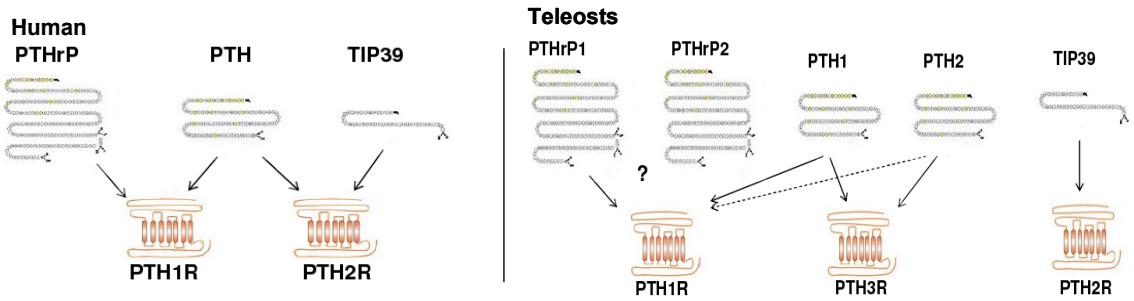

Figure 9: Comparison of the PTH/PTHrP receptor peptide potency in humans and teleost fish. Arrows indicate activation of a receptor by a ligand. The dotted arrow indicates the low-potency activation of the PTH/PTHrP receptor by PTH2 in fish (Gensure and Juppner 2005).

In teleost fishes, homologues genes of *PTH1R* and *PTH2R* were also described and a third receptor (*PTH3R*) was identified. They seem to have some similar affinity to

peptide than the human receptors, suggesting functional conservation of this system across evolution (Rubin, Hellman et al. 1999; Rubin and Juppner 1999). PTH3R is closely related to PTH1R and is also activated by PTH1, PTH2 and PTHrPA (Figure 9) and was proposed to be a fish specific duplication of *PTH1R* (Rubin and Juppner 1999; Gensure and Juppner 2005). Similar to the mammalian PTH2R, the teleost homologue receptor is also activated by the TIP39 peptide (Gensure and Juppner 2005) (Figure 9).

The duplicate PTH-family members (*PTH1* and *PTH2* and *PTHrPA* and *PTHrPB*), the novel *PTH-L* and the different receptor ligand-binding profile in fishes and humans (Figure 9) suggest multiple physiological functions in vertebrates which remain to be fully explored.

1.4.3.4.1. Avian Calcium Metabolism

In birds, as in the other vertebrates, calcium and phosphorus are essential for several biological processes. Deficiencies in these minerals can lead to skeletal abnormalities such as tibial dyschondroplasia, which is a common cause of deformity, lameness and mortality (Edwards 2000). Avian egg laying and shell calcification move approximately 5.7g of calcium carbonate ($CaCO_3$) per egg, containing about 2.3g of net calcium, which introduces an additional calcium demand compared to other classes of vertebrates (Sugiyama and Kusuhara 2001; Bar 2008). The eggshell formation requires high levels of calcium, equivalent to 10% of entire body calcium. In the case of some domestic hens, this value could be multiplied by 300, per year, which corresponds to more than 20 times a hen's total body calcium (Sugiyama and Kusuhara 2001). To support this addicional demand, 60-75% of calcium comes from dietary source and the remaining 25-40% from skeletal stores (Mueller, Schraer et al. 1964).

Birds have different type of bones. The bones involved in structural integrity are the cortical and trabecular bones, both formed of lamellar bone during growth. When a hen reaches sexual maturity, a third type of bone is formed, the medullary bone (MB). The MB is a specific tissue of female birds and crocodilian reptiles, filling 1.3 to 29.2% of marrow cavities. It seems to have a non-structural function and its formation is stimulated by androgens and estrogens, accompanying the maturation of the ovarian follicles (Sugiyama and Kusuhara 2001; Sugiyama, Sakurai et al. 2004; Bar 2009). During the lifecycle, bone undergoes a constant process of remodelling, in which osteoclast cells resorb areas of bone and are then replaced by osteoblasts that deposit new bone. During ovarian follicle maturation, the function of osteoblasts changes from forming lamellar cortical bone to producing MB in spicules within the medullary cavities, especially in the

leg bones. The humerus is a pneumatic bone *(cavity is filled with air)*, in response to a flight adaptation, but in some hens the MB can completely fill in the cavity (Figure 10) (Whitehead 2004).

This type of bone is formed in response to the egg shell formation, which occurs during the night when the supply of calcium from the digestive system is low. Thus, in a break of dietary calcium, a high proportion of calcium comes from MB resorption (Bar 2009). This calcium demand leads to a heave in osteoclastic activity, however, osteoclasts are not specific to MB and the resorption also occurs at exposed structural bone surfaces (Whitehead 2004). This explains why bone content may remain constant, or even increase, when structural bone content declines, resulting in a net resorption of structural bone. This can give rise to a risk of bone fracture, a osteoporotic structural bone profile (Whitehead and Fleming 2000). However, this process is reversed when the hen goes out of lay. MB gradually disappears and structural bone formation recommences, the osteoblasts replace the osteoclasts and the regeneration of MB begins (Whitehead and Fleming 2000; Whitehead 2004).

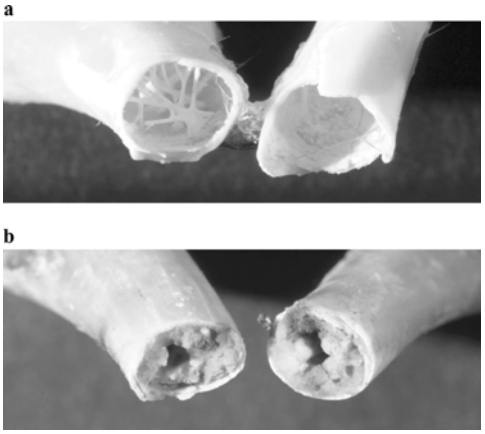

Figure 10: Humerus of laying hen showing (a) normal pneumatised internal cavity and (b) cavity filled with medullary bone (taken from (Whitehead 2004))

The mechanism behind this different activity of bone turnover is attributed mainly to estrogens (Whitehead and Fleming 2000; Bar 2009). It seems that the rise in circulating estrogens at the maturity has an inhibitory effect on osteoclast function and a stimulatory effect on osteoblasts, increasing medullary bone growth instead of structural bone. The opposite scenario occurs when estrogens levels decline (Whitehead and Fleming 2000).

The process that causes laying hen osteoporosis contrasts with that of human postmenopausal osteoporosis, in which the decline in estrogens suppresses the structural bone formation and increases bone resorption. So far, there is a lack of understanding in these processes of calcium homeostasis. The calcium balance system has been vastly studied in mammals, however, several functional aspects remain obscure, being a subject

of high interest by the pharmaceutical industry. Along with estrogens, the PTH paradox has raised many questions in the bone field. What are the critical steps in the post-PTH1R activation promoting bone resorption or mineralisation? Are other receptors involved? Is it possible produce new active therapeutic agents (Potts and Gardella 2007)? The need of other model organisms is required to answer the contradictions. Non-mammalian vertebrates, such as fishes, amphibians and birds are good alternatives. They seem to contain a functional system similar to humans and can provide novel clues about the origin, structure, regulation and function of the PTH-family members and their receptors.

1.5. Aim and outline of this thesis

The information about PTH-family members, mainly the novel member *PTH-L*, is very scarce. From sequence information and studies on fish they share a common ancestor, activate the same receptors and stimulate calcium transport. However it is not known if PTH-L features are conserved across the vertebrates and if the protein product of this gene has other functions or even where it is produced. Within the context of the comparative approach, the chicken is an excellent model to study the development of physiological systems, in particular those related to calcium storage and utilization and skeletal development and ossification.

The objective of this thesis is to take advantage of the growing genomic information on chicken and the advantages of this experimental model in relation to development and calcium regulation to investigate the origin and function of PTH-L.

The specific aims are: a) localize the expression of *PTH-L* in tissues and over time during development; b) study its biological activity; c) identify its receptor(s) and d) analyse the role of PTH-L during development.

This thesis is organized as follows:

In **Chapter II** the molecular cloning of the all members and different isoforms of PTH-family in chicken and *Xenopus* is described for the first time. Through multiple sequence alignment, phylogenetic and gene-linkage analysis, the different PTH-family members in different organisms were compared. This chapter also describes the tissue distribution of PTH-family members and their functional role in calcium flux using chicken chorionallantois membrane and *Xenopus* skin.

The **Chapter III** reports the receptors for the PTH-family in chicken, establishing their expression, evolution and functional pathway. This chapter complements the knowledge about this family, ligand binding profile and a different PTH-family receptors evolution is suggested to *avian* lineage. The receptors expression is accessed by RT-PCR in adult and embryos tissues, the different intracellular pathways are analysed in the presence o PTH-family ligands, and *in silico* studies compare the sequences in different organisms.

In **Chapter IV** the PTH-family members tissue expression is reported during chicken ontogeny, taking a step forward in characterisation of each transcript. The RT-PCR, *in situ* hybridization and optical projection tomography techniques show the presence of this family also during embryogenesis and a new specific expression pattern to each family member is also revealed.

The **Chapter V** brings about the PTH-family action during embryo/skeletogenesis. The knock-down studies suggest a functional role in cartilage development also to the novel tetrapod PTH-L, contributing to characterise the functional role of this family.

In the **Chapter VI** the main results are discuss and future perspectives outlined.

CHAPTER II

Gene Structure, Transcripts and Calciotropic Effects of the PTH-family of Peptides in *Xenopus* and Chicken

In: João C.R. Cardoso, Ana S. Gomes, Juan Fuentes, Deborah M. Power and Adelino V.M. Canário. BMC Evolutionary Biology (2010) 10(1): 373.

Abstract

Parathyroid hormone (PTH) and PTH-related peptide (PTHrP) belong to a family of endocrine factors that share a highly conserved N-terminal region (amino acids 1-34) and play key roles in calcium homeostasis, bone formation and skeletal development. Recently, PTH-like peptide (PTH-L) was identified in teleost fish raising questions about the evolution of these proteins. Although *PTH* and *PTHrP* have been intensively studied in mammals their function in other vertebrates is poorly documented. Amphibians and birds occupy unique phylogenetic positions, the former at the transition of aquatic to terrestrial life and the latter at the transition to homeothermy. Moreover, both organisms have characteristics indicative of a complex system in calcium regulation. This study investigated PTH-family evolution in vertebrates with special emphasis on *Xenopus* and chicken.

The *PTH-L* gene is present throughout the vertebrates with the exception of placental mammals. Gene structure of *PTH* and *PTH-L* seems to be conserved in vertebrates while *PTHrP* gene structure is divergent and has acquired new exons and alternative promoters. Splice variants of *PTHrP* and *PTH-L* are common in *Xenopus* and chicken and transcripts of the former have a widespread tissue distribution, although *PTH-L* is more restricted. *PTH* is widely expressed in fish tissue but from *Xenopus* to mammals becomes largely restricted to the parathyroids. The N-terminal (1-34) region of PTH, PTHrP and PTH-L in *Xenopus* and chicken share high sequence conservation and the capacity to modify calcium fluxes across epithelia suggesting a conserved role in calcium metabolism possibly via similar receptors.

The parathyroid hormone family contains 3 principal members, *PTH*, *PTHrP* and the recently identified *PTH-L*. In teleosts there are 5 genes which encode *PTHrP* (2), *PTH* (2) and *PTH-L* and in tetrapods there are 3 genes (*PTHrP*, *PTH* and *PTH-L*), the exception is placental mammals which have 2 genes and lack *PTH-L*. It is hypothesized that genes of the PTH-family appeared at approximately the same time during the vertebrate radiation and evolved via gene duplication/deletion events. *PTH-L* was lost from the genome of eutherian mammals and *PTH*, which has a paracrine distribution in lower vertebrates, became the product of a specific endocrine tissue in Amphibia, the parathyroids. The PTHrP gene organisation diverged and became more complex in vertebrates and retained its widespread tissue distribution which is congruent with its paracrine nature.

2.1. Introduction

Parathyroid hormone (PTH) and PTH-related peptide (PTHrP) belong to a family of endocrine factors with a highly conserved N-terminal region (amino acids 1-34), which accounts for their overlapping functions in calcium homeostasis (Potts 2005; Canario, Rotllant et al. 2006; Guerreiro, Renfro et al. 2007). In mammals, single copy PTH and PTHrP genes are proposed to share common ancestry (Gensure, Gardella et al. 2005; Potts 2005; Guerreiro, Renfro et al. 2007) an idea reinforced by the identification of duplicate orthologous genes, *PTH1* and 2, and *PTHrP1* and 2 in teleost fishes (Danks, Ho et al. 2003; Gensure, Ponugoti et al. 2004; Canario, Rotllant et al. 2006) which underwent a specific genome duplication (Jaillon, Aury et al. 2004). However, the recent identification of a novel PTH-like (*PTH-L*) gene in teleosts throws into question previous evolutionary models for this gene family (Canario, Rotllant et al. 2006).

In mammals, *PTH* is a product of the PTGs and pre-pro-PTH is processed to liberate the biologically active mature 84 amino acid hormone, which regulates serum calcium through its direct actions in bone and kidney counteracting the action of calcitonin (Habener, Kemper et al. 1976; Potts 2005). In contrast, PTHrP is a pluripotent hormone which acts via intracellular, paracrine and endocrine pathways and regulates cell growth and differentiation, bone development and lactation, and embryonic and fetal development and survival (Moseley and Gillespie 1995; Philbrick, Wysolmersky et al. 1996; Minina, Kreschel et al. 2002; Safer, Ray et al. 2007). Tissue specific proteolytic processing of PTHrP occurs and generates at least three active fragments (Moseley and Gillespie 1995; Ingleton and Danks 1996; Gensure, Gardella et al. 2005), of which only the N-terminal (1-36) fragment has a cognate family 2 G-protein coupled receptor, PTH1R, which also binds PTH (Juppner, Abou-Samra et al. 1991). Moreover, alternative promoter utilization and exon splicing generates several different human *PTHrP* isoforms which range in length from 139-173 amino acids (Orloff, Reddy et al. 1994; Gensure, Gardella et al. 2005). In mammals, a second receptor (PTH2R) is activated by PTH and tuberoinfundibular peptide 39 (TIP39), while in teleost fish PTH2R is only activated by TIP39 (Usdin, Hoare et al. 1999; Papasani, Gensure et al. 2004). Moreover, in teleosts a paralogue of tetrapod PTH1R (designated PTH3R) with affinity for PTHrP also exists (Rubin, Hellman et al. 1999; Rubin and Juppner 1999; Rotllant, Guerreiro et al. 2005; Rotllant, Redruello et al. 2005; Rotllant, Guerreiro et al. 2006).

PTH and *PTHrP* have been intensively studied in mammals but their function in other vertebrates is poorly documented. In amphibians, PTH/PTHrP receptors have been characterized and an immunoreactive PTHrP-like peptide with widespread tissue

distribution has been detected (Danks, McHale et al. 1997; Bergwitz, Klein et al. 1998). In chicken, *PTH* and *PTHrP* homologues have also been isolated and are involved in chondroblast and osteoblast differentiation, although their role in calcium transport mechanisms is poorly understood (Rosenberg, Pines et al. 1989; Packard, Clark et al. 1998; Ieda, Takahashi et al. 2000; Medill, Praul et al. 2001; Zhao, Brauer et al. 2002; Zuscik, O'Keefe et al. 2002). Amphibians and birds occupy unique phylogenetic positions, the former at the transition of aquatic to terrestrial life, the latter at the transition to homeothermy, and both organisms have a complex system in calcium regulation. The presence of a PTG in frogs (not present in fish) coupled with their terrestrial/aquatic environment and the occurrence in birds of a hollow skeleton and heavily calcified eggs are examples of physiological/structural processes which influence calcium and phosphorus requirements.

The identification of new PTH-family members in *Xenopus* sp. (Amphibia) and chicken, *Gallus gallus* (Aves) by *in silico* analysis of public databases and gene cloning is reported. Gene structure, gene linkage, alternative splicing and tissue specific transcription are also characterised. The calciotropic activity of *Xenopus* and chicken N-terminal (1-34) peptides of PTH-family members *in vitro* are established using *Xenopus* skin and chicken chorionallantois membranes. Finally a model of *PTH* gene family evolution is proposed and discussed in the context of functional divergence.

2.2. Methods

2.2.1. Animals and tissue collection

Adult *Xenopus laevis* were purchased from Xenopus Express (Diaz Berenguer, Cabeza Mora et al.) and maintained at 22°C. Adult chickens (*Gallus gallus*) were supplied by a local farm. Frogs and chickens were anesthetized with diethyl ether and euthanized by double pithing and decapitation, respectively. Fertile white leghorn chicken eggs were obtained from Quinta da Freiria (Serpa, Portugal) and kept in humid conditions in an automatic incubator (Brinseca OCTAGON 40) at 37.5 ºC with gentle rotation. Tissues were collected, immediately frozen in liquid nitrogen and stored at -80ºC. All animal experiments were performed in accordance with Portuguese legislation under a “Group-1” licence from the Direcção-Geral de Veterinária, Ministério da Agricultura, do Desenvolvimento Rural e das Pescas, Portugal.

2.2.2. *In silico* identification and validation of PTH-like transcripts

Putative PTH-like genes were identified in the amphibian (*Xenopus tropicalis)* and chicken (*Gallus gallus*) genome and EST databases by sequence similarity searches using human PTH (AAH96144.1) and PTHrP (AAA60221) and *Takifugu rubripes* PTHA (CAG26460.1), PTHB (CAG26461.1), PTHrPA (CAB94712.1), PTHrPB (CAG26459.2) and PTH-L (CAG26462.1) and the default settings of tBLASTn (Altschul, Gish et al. 1990). Amphibian and chicken genomes were accessed via Ensembl (http://www.ensembl.org), Xenbase (http://www.xenbase.org) and NCBI (http://www.ncbi.nlm.nih.gov/), respectively.

Table 1: Primer pairs (same prefix ending in fw or rv) used to amplify the *Xenopus* and chicken PTH-family members. $ indicates primers for RT-PCR and * indicates primers for q-PCR. For *Xenopus* PTHrP the same forward primer was used for each pair.

	Xenopus laevis	**Chicken**
PTH	*PTHfw: aggagacgggctgtgagtgag$*	*PTHfw: atgacttctacaaaaaatctg$*
	PTHrv: tcattggatgccaggcttta$	*PTHrv: tggcttagttttaaagagta$*
	*PTH2fw: tcagatgaagttacaggac**	*PTHfw: gcataaccttggagagcatcg**
	*PTH2rv: cttagtgctatgcctatg**	*PTHrv: cctctgggtcctggcatc**
PTHrP	*PTHrPfw: cagtatctccacgacaaagg*$*	*PTHrP(1-139)fw: ctgagagcccagtcttgga$*
	PTHrP(1-131)rv:ttacctgtaatctaattcttcca$	*PTHrP(1-139)rv: gggtaacaatttcagtaact$*
	PTHrP(1-144)rv cgggtgccgctcatctgc$	*PTHrP(1-141)5utrAfw: gaagggagtagcacctgggc$*
	*PTHrPrv: tggtggcagggagtaag**	*PTHrP(1-141)5utrBfw: ggcacctgcttttaaaaccc$*
		PTHrP(1-141)5utrCfw: gctaacagaggaactgcgac$
		PTHrP(1-141)5utrDfw: aggactgacccctcctttcc$
		PTHrP(1-141)rv: gatcccctctactgatcttcc$
		*PTHrP(1-139)fw: agcaaagcctggaaaacg**
		*PTHrP(1-139rv: gtggaaaagatacagcagaattacc**
		*PTHrP(1-141)5utrAfw: caggcttgcggtgaggcta**
		*PTHrP(1-141)5utrArv: gcgaaactccactgctgaaag**
		*PTHrP(1-141)5utrBfw: tgacccctcctttccttgc**
		*PTHrP(1-141)5utrBrv: ggcacagaataactcagaagaaac**
		*PTHrP(1-141)5utrCfw: cagaggaactgcgacgaacaac**
		*PTHrP(1-141)5utrCrv: gcgaaactccactgctgaaag**
		*PTHrP(1-141)5utrDfw: ggcacctgcttttaaaaccc**
		*PTHrP(1-141)5utrDrv aaggttttgatgaaagataggaatcc**
PTH-L	*PTH-Lfw: gagagatcagttgcagagg$*	*PTH-Lfw: gaacgacaagagaaggaaag$*
	PTH-Lrv: tgaaggatcccgctccatt$	*PTH-Lrv: ctgcttcatcgggtttga$*
	*PTHLfw: ttgaagaaataaatcgccagag**	*PTHLfw: gataaggcgagggcatttcaag**
	*PTHLrv: atgctgctgattctttgctgt**	*PTHLrv: cctgctgctggctgtgtg**
r18S	*18s fw tgacggaagggcaccaccag**	*18s rv aatcgctccaccaactaagaacgg**

EST sequences were retrieved from NCBI and BBSRC ChickEST databases (http://www.chick. manchester.ac.uk/). Isoforms of *Xenopus* and chicken transcripts were

named according to the size of the deduced mature protein and the length of their 5'UTR region (A to D). Puffer fish (*Takifugu rubripes*) and human (*Homo sapiens*) genome assemblies available in Ensembl and the human dbEST NCBI database were also interrogated with *Xenopus* and chicken PTH-family members to identify potential novel isoforms.

Validation of transcripts was done by specific PCR amplifications using *Xenopus* and chicken cDNAs as template, the primers listed in Table 1 and the number of thermocycles and annealing temperatures adjusted for each amplicon. *Xenopus* reactions were cycled 40 times with annealing temperatures of 59ºC for PTH, 55ºC for PTHrPA, 58ºC for PTHrPB and 57ºC for PTH-L. For chicken the annealing temperatures and cycles were, respectively, 53ºC and 30 for PTH, 55ºC and 35 for PTHrP(1-139), 58ºC and 35 for PTHrP(1-141) and 55ºC and 40 for PTH-L. All PCR products were analysed on 1.5% agarose gel and sequenced to confirm their identity.

2.2.3. RNA extractions and quantitative gene expression

Total RNA (tRNA) extracted from adult frog and chicken tissues using Tri Reagent (Sigma Aldrich, Spain) was treated with 1U DNase (DNA-free Kit, Ambion, UK) for 30 min at 37ºC. DNase treated tRNA (500ng) was denatured at 65ºC for 5 min, quenched on ice for 5 min and used for cDNA synthesis in a 20 μl reaction volume containing 10 ng of pd(N)6 random hexamers (GE Healthcare, UK), 2mM dNTPs, 100U of MMLV-RT and 20U RNasin® Plus RNase inhibitor. cDNA was synthesized for 10 min at 20ºC followed by 50 min at 42ºC and 72ºC for 5 min.

Quantitative real-time PCR (q-PCR) amplifications of *Xenopus* and chicken cDNAs used the primers listed in Table 1 designed with Primer Premier and Beacon Design software (Premier Biosoft Int., Palo Alto, CA). Triplicate reactions (20μl final volume) containing 1μl of template cDNA and 1pmol of each primer were prepared and reactions repeated twice using Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA) and a Bio-Rad iClycler iQ thermocycler system (software version 3.1.7050, Bio-rad, Life Science Group, USA). The thermocycle consisted of an initial step at 95ºC for 10 min followed by 55 cycles of 95ºC for 30 sec, 20 sec at an appropriate temperature for annealing of each primer pair, and 72ºC for 30 sec. Annealing temperatures were 60ºC for r18S; 56ºC, 59ºC and 53ºC for the *Xenopus* PTH, PTHrP and PTH-L, respectively; and for the chicken amplicons 53ºC for PTHrP(1-141)*5utrB*; 55ºC for PTHrP(1-139); 57ºC for PTH, PTH-L and PTHrP(1-141)*5utrC*; 58ºC for PTHrP(1-141)*5utrD*; and 60ºC for PTHrP(1-141)*5utrA*. Melting curves were performed to detect nonspecific products and primer dimers. PCR products were quantified relative to a standard curve constructed

using serial dilutions of linearized DNA plasmid of the target transcript. Genomic contamination was monitored by including tRNA samples without MMLV-RT and r18S was used as the internal quantitative control for normalization. Relative gene expression was calculated as: number of copies (NC)= (A x 6.022 $x10^{23}$)/(Bx1$x10^{9}$x650 kDa), where A is the template quantity (ng of vector plus insert), B the template length (bp vector plus insert), and 650 kDa is the average weight of a base pair according to (Martyniuk, Kroll et al. 2009).

2.2.4. Sequence comparisons and phylogenetic analysis

Multiple sequence alignments of PTH-family prepro- and mature proteins were performed with ClustalX using the following parameters: Gonnet series matrix, Gap opening penalty 10, Gap extension 0.2 (Thompson, Gibson et al. 1997). Alignments were displayed in GeneDoc (www.psc.edu/biomed/genedoc), manually edited and percentages of sequence identity and similarity calculated. Phylogenetic analysis was performed using both Neighbor Joining and Maximum Parsimony Methods (Saitou and Nei 1987) with 1000 and 100 bootstrap replicates, respectively, using MEGA 3.1 software (Kumar, Tamura et al. 2004).

2.2.5. Gene organization and linkage analysis

The gene organization of the *Xenopus* and chicken PTH-like members was deduced using Spidey software (mRNA-to-genomic alignment; http://www.ncbi.nlm.nih.gov/IEB/Research /Ostell/Spidey) with the aid of Ensembl *in silico* gene annotation and the predicted structures were manually edited using intron/exon splice boundary consensus sequences (AG/GT) and by comparison with available EST data. The immediate gene environment of the PTH, PTHrP and PTH-L genes in *Xenopus* was assessed from scaffold annotation in Ensembl and the chicken homologue regions using Mapview (http://www.ncbi.nlm.nih.gov/mapview). To verify if conservation of the PTH-L genome region in amphibian and chicken genomes also exists in human and puffer fish, a search for homologues of neighbouring genes was performed.

2.2.6. Electrophysiological measurements and unidirectional calcium fluxes

Previous studies established that PTH promotes calcium transport across the frog skin (Stiffler, Yee et al. 1998) and chicken chorionallantois membranes (CAMs) (Packard, Clark et al. 1998) thus providing assays to test the activity of the new PTH-family peptides. The effect of *Xenopus* and chicken PTH(1-34), PTHrP(1-34) and PTH-L(1-34) (Genemed Synthesis, Inc., San Antonio, Texas, USA) on calcium transport was assessed *in vitro* using Ussing chambers with adult *Xenopus* abdominal skin and CAMs from

chicken embryos of 16 to 18 days (stages 42 HH and 44 HH, respectively; Hamburger and Hamilton 1992). Human PTH (1-34) (Bachem, Germany) and salmon Luteinizing hormone-releasing hormone (LHRH, Bachem) were used as positive and negative controls, respectively.

The *Xenopus* experiments were carried out at 22-23ºC in Ringers solution [2.4 mM $NaHCO_3$; 113.8 mM NaCl; 1.9 mM $KC_2H_3O_2$; 1 mM $CaCl_2$; 2.1 mM $NaC_2H_3O_2$; 0.5 mM $Mg(C_2H_3O_2)_2$ and 5 mM glucose] at pH 8.1 with oxygenation provided by atmospheric air. The chicken experiments were carried out at 37ºC using a standard bathing solution [130mM NaCl; 1mM $MgSO_4$; 2mM $CaCl_2$; 8mM KH_2PO_4; 15mM glucose (Graves, Dunn et al. 1986)] at pH 7.4 maintained by gassing with a mixture of 5% CO_2 in O_2.

The *Xenopus* and chicken membranes were pinned over the circular aperture of Ussing chambers (1cm^2) and 8 ml of saline solution was added to each half-chamber. Epithelial preparations were stabilized for 30 min and the saline solution was replaced before the addition of radioactive labelled $^{45}Ca^{2+}$ (0.75 μCi/ml $CaCl_2$; GE Healthcare, UK) to the mucosa/chorion side. Time zero was established 15 min after $^{45}Ca^{2+}$ addition. Fluid samples (400μl) were collected every 30 min over a total of two hours from the serosa/allantoic side and the first 2 samples served as controls prior to peptide (10 nM) addition. The volume sampled was replaced by an equal amount of saline and replicate 200μl samples of the mucosa/chorion side were used to calculate calcium specific activity by counting in a liquid scintillation counter (Beckamn LS 6000IC, USA). All radiotracer experiments were performed under short circuit conditions.

Bio-electrical variables were recorded with a DVC-1000 voltage-clamp amplifier (WPI, Sarasota, US) by means of Ag-AgCl electrodes connected to the chamber by agar bridges (2M KCl/ 3% agar) and data was collected via a Data-Trax acquisition system (WPI, Sarasota, US) connected to a personal computer. At the start of experiments the trans-epithelial potential (Vt, mV) was recorded to check tissue integrity and then short circuited (Vt=0) for subsequent experiments. Short circuit current (Isc, μAmp/cm^2) was constantly recorded and the current deflections produced by 3mV pulses once every minute were used for calculation of tissue resistance (*R*t, Ω.cm^2) using Ohm's law.

Calcium fluxes were calculated using the equation: $J_{in}^{Ca2+} = \Delta\left[{}^{45}Ca\right]_{BI}(1/SA_{AP})(volume_{BI})/[(time)(area)]$, where $\Delta\left[{}^{45}Ca\right]_{BI}$ represents the increase in radioactivity in the basolateral side (BI) half-chamber and SA_{Ap} the apical side (Ap) specific activity (cpm/nmol) (Fuentes, Figueiredo et al. 2006). Data is presented as mean ± standard error of the mean (SEM). A paired Student's t-test was used to test the effect of peptide on calcium fluxes and one-way ANOVA was used to test the effect of peptide

on electrophysiological measurements (Isc e Rt) using SigmaStat v.3.11 (Systat software, Inc., USA). The significance level was 5%.

2.3. Results

2.3.1. Xenopus and chicken PTH-family members

Homologues of vertebrate *PTH*, *PTHrP* and *Takifugu rubripes PTH-L* genes were identified or deduced *in silico* using the *Xenopus* and chicken genomes (Table 2) and validated by cDNA cloning and sequencing (Figure 11, Figure 12 and Figure 13). The *Xenopus* and chicken PTH-family members shared at least 30% amino acid sequence similarity to the *Takifugu* mature proteins and 58% to the (1-34) N-terminal amino acid residues. Sequence comparison of *Xenopus* and chicken PTH-family members with other vertebrate homologues revealed that specific sequence motifs are conserved (Figure 14). For example, the cleavage sites located before the mature protein in human pre-pro-PTH and PTHrP were conserved (Figure 11 and Figure 12).

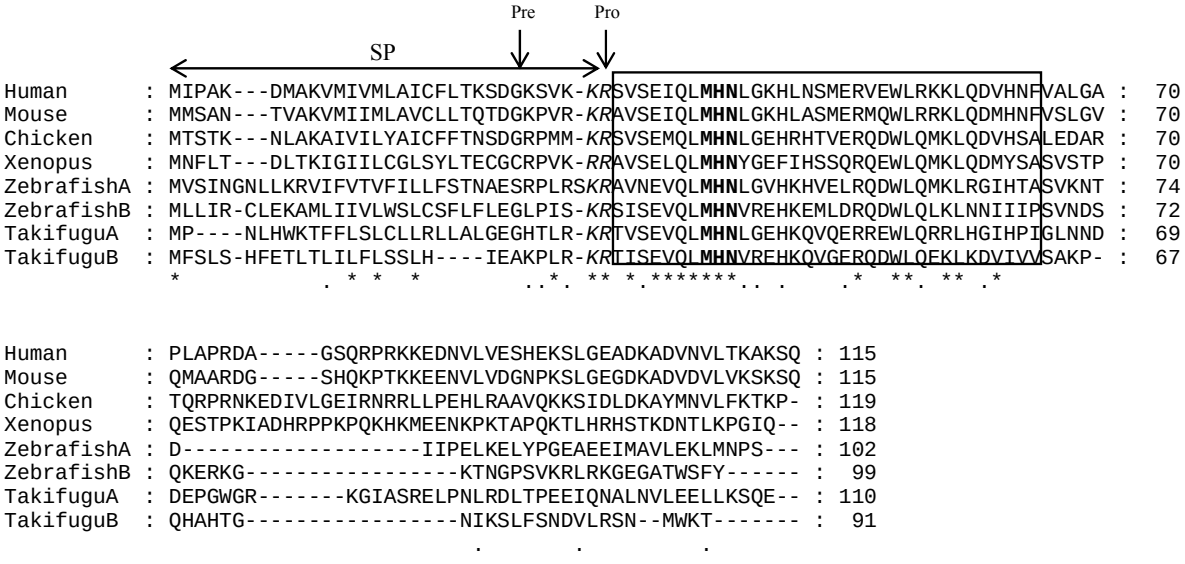

Figure 11: Multiple sequence alignment of vertebrate PTH. The signal peptide (SP) is indicated by a double arrow and the 1-34 mature peptide is boxed. Potential proteolytic cleavage sites are in bold and italics and the Pre and Pro sites are indicated. The M-H-N amino acid motif is indicated in bold. Amino acid conservation is denoted by "*" and accession number of the sequences used are indicated in Figure 14.

Characteristic proteolytic sites within the human PTHrP protein, which generate three distinct peptides were also conserved: N-terminal PTHrP(1-36), mid-region PTHrP (38-94) and C-terminal PTHrP (107-139) (Philbrick, Wysolmersky et al. 1996) (Figure 12).

The first 10 N-terminal amino acids, involved in the calciotropic action of the hormones is the most highly conserved domain. Specific motifs such as, M-H-N in PTH, and L-H-D in tetrapod PTHrP and the amino acids important in receptor-binding in mammals, L^{24} and L^{28} are also conserved. Additional conserved residues in *Xenopus* and chicken PTH-family members are V^{2}, Q^{6}, H^{9} and R^{20} (Figure 14), suggesting they are important in peptide function from fish to mammals.

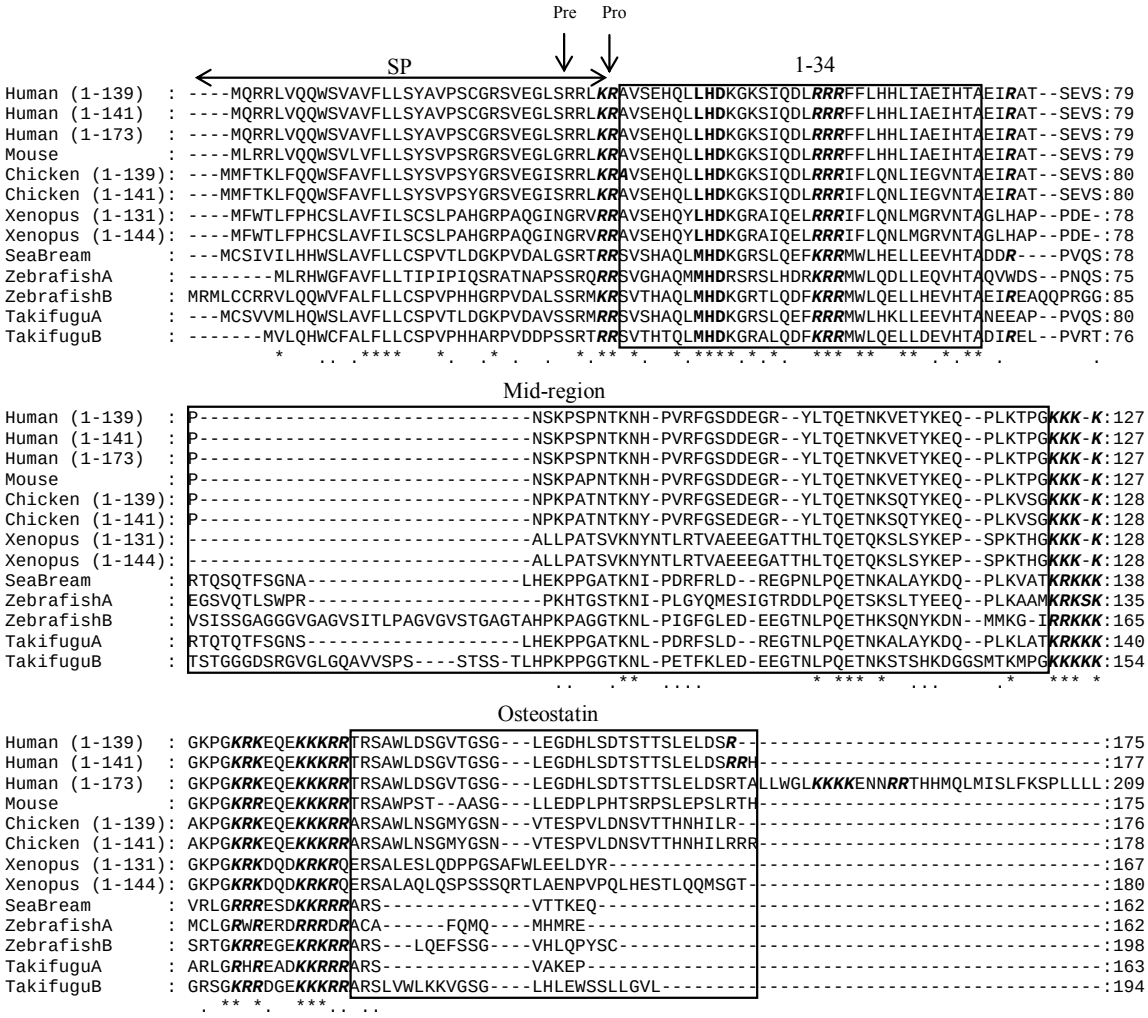

Figure 12: PTHrP amino acid multiple sequence alignment. The signal peptide (SP) is indicated by a double arrow and the three potential peptides (1-34PTHrP, mid-region and osteostatin) generated from the human precursor are indicated within boxes. The alignment includes various human, chicken and *Xenopus* PTHrP isoforms which are annotated according to the length of the mature protein sequence. Potential cleavage sites are in italics and bold and the Pre and Pro cleavage sites are indicated by arrows. The tetrapod L-H-D and the teleost M-H-D motifs are annotated in bold. The two lamprey PTH-like sequences were not included in the alignment since only the mature peptide region was characterized. Amino acid conservation is denoted by "*" and accession numbers of the sequences used are described in Figure 14.

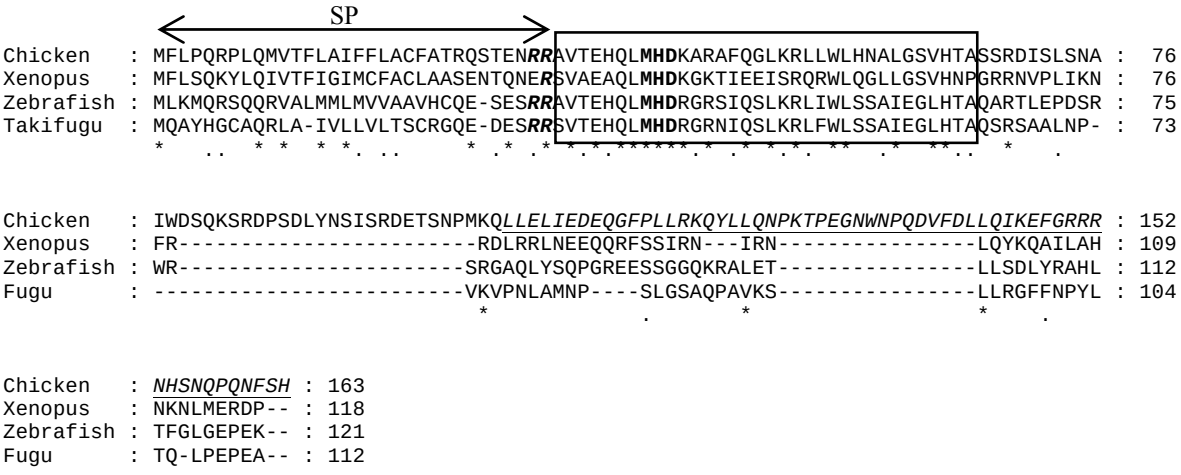

Figure 13: PTH-L amino acid multiple sequence alignment. The signal peptide (SP) is indicated by a double arrow and the potential mature PTH-L peptide is within a box. The potential M-H-D motif previously identified in the *Takifugu* precursor is in bold and potential proteolytic cleavage sites within the signal peptide sequence are in bold and italics. Sequences underlined and in *italics* have been predicted *in silico* but were not confirmed by RT-PCR. Amino acid conservation is denoted by "*" and accession numbers are indicated in Figure 14.

Table 2: *Xenopus* and chicken parathyroid family gene and transcript data. Accession numbers (GenBank and Ensembl IDs) of nucleotide sequences, gene scaffolds and tissue of origin of EST of the vertebrate PTH-family members. The accession number of the predicted genes is indicated or when not available their putative location in genome regions in ENSEMBL assembly (*Xenopus* Scaffold_235 and Scaffold_169; chicken Contig 68.24) or NCBI assembly if available (chicken NC 006088.2 and NC_006092.2 indicated by a $^{+}$). Accession numbers for ESTs identified in this study are indicated in italics and highlighted with an * are the GeneBank accession numbers of isolated cDNAs.

	Xenopus			**Chicken**		
	Gene	**Transcripts**	**ESTs origin**	**Gene**	**Transcripts**	**ESTs origin**
PTH	Scaffold_235	FM955441*		NC 006088.2^{+}	NM_205452	
				ENSGALG00000017295	*CV890868* *CV041147*	Mix tissues (brain, ultimobranchial, PTGs, cecal tonsil, primordial germ cells)
PTHrP	ENSXETG00000001307	FM955442*		NC_006092.2^{+}	NM_205338	
		CR437266	tailbud (stage 28-30)	ENSGALG00000005358	*BU384898*	trunks (stage 36)
		CR433007	tailbud (stage 28-30)		*BU252785*	limbs (stage 36)
					BU252877	limbs (stage 36)
					BM489067	Muscle (breast and leg); epiphyseal growth plate
					ENSGALEST T000000309 72	
PTH-L	Scaffold_169	*AL775245*	gastrula (stages 10.5- 12)	Contig 68.24^{+}	FM955443*	
		AL964863	gastrula (stages 10.5- 12)	NW_001471609		
		AL965929	gastrula (stages 10.5- 12)			
		BX750389	gastrula (stages 10.5- 12)			
		BX764109	gastrula (stages 10.5- 12)			
		CN076482	brain and spinal cord			
		CN076481	(tadpoles stage 58-64)			

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PTH
Human       SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNF 100%
Mouse       AVSEIQLMHNLGKHLASMERMQWLRRKLQDMHNF  97%
Chicken     SVSEMQLMHNLGEHRHTVERQDWLQMKLQDVHSA  88%
Xenopus     AVSELQLMHNYGEFIHSSQRQEWLQMKLQDMYSA  82%
ZebrafishA AVNEVQLMHNLGVHKHVELRQDWLQMKLRGIHTA  70%
ZebrafishB SISEVQLMHNVREHKEMLDRQDWLQLKLNNIIIP  70%
TakifuguA   TVSEVQLMHNLGEHKQVQERREWLQRRLHGIHPI  76%
TakifuguB   TISEVQLMHNVREHKQVGERQDWLQEKLKDVIVV  73%

PTHrP
Human       AVSEHQLLHDKGKSIQDLRRRFFLHHLIAEIHTA 100%
Mouse       AVSEHQLLHDKGKSIQDLRRRFFLHHLIAEIHTA 100%
Chicken     AVSEHQLLHDKGKSIQDLRRRIFLQNLIEGVNTA  91%
Xenopus     AVSEHQYLHDKGRAIQELRRRIFLQNLMGRVNTA  88%
ZebrafishA SVGHAQMMHDRSRSLHDRKRRMWLQDLLEQVHTA  85%
ZebrafishB SVTHAQLMHDKGRTLQDFKRRMWLQELLHEVHTA  76%
TakifuguA   SVSHAQLMHDKGRSLQEFRRRMWLHKLLEEVHTA  85%
TakifuguB   SVTHTQLMHDKGRALQDFKRRMWLQELLDEVHTA  85%

PTH-L
Takifugu    SVTEHQLMHDRGRNIQSLKRLFWLSSAIEGLHTA 100%
Chicken     AVTEHQLMHDKARAFQGLKRLLWLHNALGSVHTA  79%
Xenopus     SVAEAQLMHDKGKTIEEISRQRWLQGLLGSVHNP  58%
Zebrafish   AVTEHQLMHDRGRSIQSLKRLIWLSSAIEGLHTA  97%
              *   * ***         *  **   *  *
```

Figure 14: Multiple sequence alignment of the *Xenopus* and chicken 1-34 PTH-family members mature peptide N-terminal region with teleost (*Takifugu* and zebrafish) and mammals (human and mouse). Conserved amino acid positions identified in all vertebrates are indicated by "*" and percentage of sequence similarity in comparison with human PTH and PTHrP and *Takifugu* PTH-L is given. The typical three amino acid motifs characteristic of each PTH-family member in positions 8 to 10 are indicated in black. % similarity to first sequence is indicated on the right. Accession number of the sequences used were: Human (PTH, AAH96144.1; PTHrP, AAA60216); Mouse (PTH, NP_065648; PTHrP, CAC39218.1); Zebrafish (PTHA, NP_998115.1; PTHB, NP_998114.1; PTHrPA, AAY87956.1; PTHrPB, AAY87957.1; PTH-L, CU856139); *Takifugu* (PTHA, CAG26460.1; PTHB, CAG26461.1; PTHrPA, CAB94712.1; PTHrPB, CAG26459.2; PTH-L, CAG26462.1).

2.3.2. Gene structure and transcript isoforms

Comparison of gene structure indicates that amphibian and chicken *PTH* and *PTH-L* gene organization is conserved and that the peptide precursors are encoded by two separate exons (E1 and E2) Figure 15. In contrast, the gene structure of PTHrP from *Xenopus* and chicken is poorly conserved and 3 exons code for the mature protein. Alternative splicing generates two distinct transcripts that differ in their 3' region and modify the predicted amino acid sequence of the C-terminal domain (Figure 12).

Transcripts - PTH

The *Xenopus PTH* gene was deduced *in silico* and revealed that exon 1 encodes the pre-pro-region of the protein and exon 2 the mature protein (Figure 15). *Xenopus PTH* cDNA was amplified by RT-PCR from the intestine (accession number FM955441)

and the predicted protein has 118 amino acids and yields a mature protein of 87 amino acids. It was not possible to establish the nucleotide sequence of the 5' and 3' UTR of *Xenopus PTH*. The gene structure of chicken *PTH* was identical to the amphibian homologue but no evidence of alternative splicing was found (Figure 15). Chicken PTH has 119 amino acids and is identical to the previously described cDNA (NM_205452, Khosla, Demay et al. 1988; Russell and Sherwood 1989). Two ESTs were identified (CV890868 and CV041147) from cDNA of a mixture of chicken tissues (whole brain, ultimobranchial gland, PTG, cecum, tonsil, and primordial germ cells) and were identical to full-length PTH (accession numbers NM_205452 and M36522).

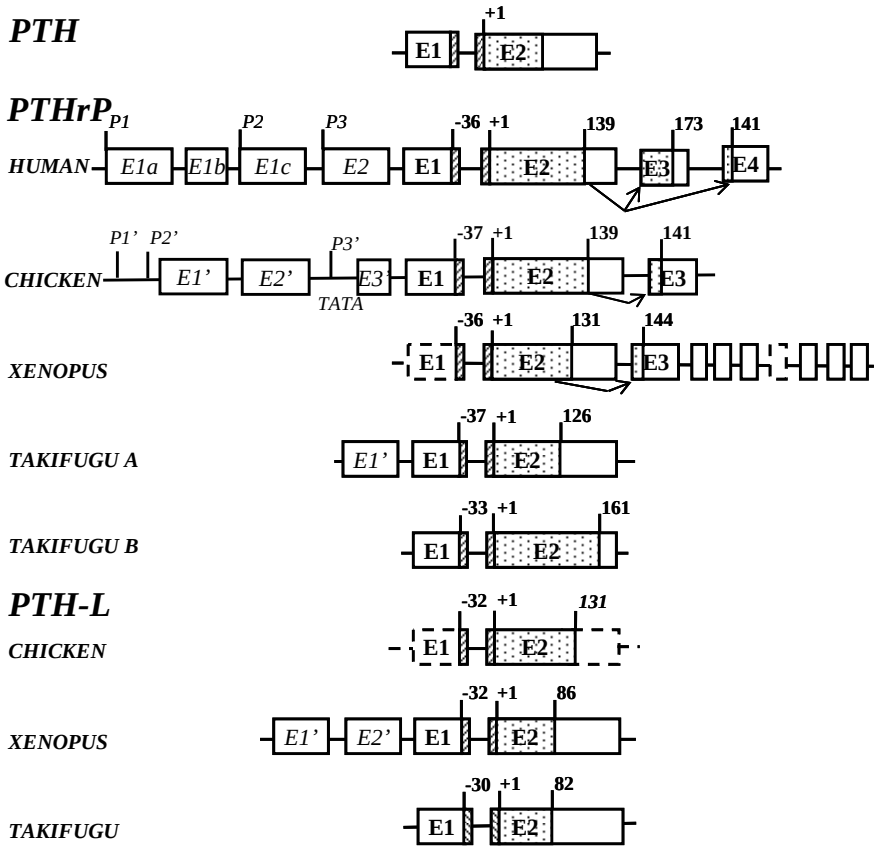


Figure 15: Gene organization of the vertebrate PTH-like family members. Exons are represented by boxes and lines indicate introns. Coding (E1 to E4) and non-coding (E1' to E'3) exons are numbered and annotated in bold and italics, respectively. Dotted-filled boxes represent the mature coding regions and black lines box the signal peptide sequence. Arrows represent alternative splice isoforms identified in *Xenopus* and chicken and previously reported in human. The general organization of the conserved vertebrate *PTH* gene structure is represented and the size of vertebrate *PTHrP* and *PTH-L* precursors is given (amino acids). The length of the chicken PTH-L precursor was predicted *in silico* and is indicated in italics. Dashed lines indicate incomplete structures that were not confirmed *in silico* or amplified by RT-PCR. The start of the mature peptide (+1) and the size of the signal peptide for all vertebrate PTH-family members is indicated. The

localization of the human PTHrP promoter regions (P1, P2 and P3) and the chicken PTHrP putative promoter sites (P1', P2' and P3') and TATA box consensus sequence within the region of P3' are shown. The figure is not drawn to scale and *Takifugu* A structure was taken from Power et al. (Power, Ingleton et al. 2000).

Transcripts - PTHrP

The deduced *Xenopus PTHrP* gene is composed of at least 10 exons (Figure 15). The first putative exon contains the pre-pro-peptide and part of the 5'UTR, although the full gene structure in this region remains to be elucidated. Exon 2 encodes the mature protein and part of the 3'UTR and yields Xenopus PTHrP(1-131) (XenPTHrP). The largest XenPTHrP transcript identified has 144 amino acids and results from intra-exon splicing of exon 2 to exon 3 as occurs in the homologue region (osteostatin) in the human PTHrP gene. Through alternative splicing, exon 3 donates 32 amino acids to the predicted protein and at least seven downstream exons generate a unique 3'UTR domain. *Xenopus PTHrP* encodes the largest 3'UTR region identified for this gene in vertebrates.

Two ESTs identified in the tailbud (stage 28-30) (accession numbers CR437266; CR433007) encode a predicted XenPTHrP of 180 amino acids which yields a mature protein of 144 amino acids [XenPTHrP(1-144)]. A second XenPTHrP isoform encoding a protein of 167 amino acids which generates a mature peptide of 131 amino acids [XenPTHrP(1-131)] was predicted from analysis of genomic sequences. The existence of XenPTHrP(1-131) transcript was confirmed by RT-PCR of bone (accession number FM955442).

The deduced chicken *PTHrP* gene is composed of at least 6 exons (Figure 15 and Figure 16). The first three exons undergo alternative splicing and contain part of the 5'UTR (Figure 16). Exon 4 encodes the remaining 5'UTR and part of the pre-pro-protein. The mature protein and part of the 3'UTR of chicken PTHrP(1-139) are included in exon 5. In common with human an intra-exon splice of exon 5 to 6 give rise to chicken PTHrP(1-141). This final exon encodes for the last 3 amino acids of PTHrP(1-141) and the 3'UTR. Several chicken PTHrP (ckPTHrP) isoforms which differed in the 5' UTR and coding region were identified amongst isolated ESTs (Table 2). Five ESTs (BU252785, BU384898, BM489067, ENSGALESTT00000030972 and BU252877) differing in their 5'UTR region, encoded a ckPTHrP of 141 amino acids (Figure 16). No ESTs for putative ckPTHrP(1-139) were identified in database searches although RT-PCR revealed it is expressed in several tissues (Figure 17). Two previously published mRNA (NM_205338 and AB175678) (Thiede and Rutledge 1990; Okabe and Graham 2004) encode ckPTHrP 1-139 and 1-141 which share an identical amino acid sequence with the exception of two extra arginine (R) residues in the latter isoform contributed by exon (E3) (Figure 15).

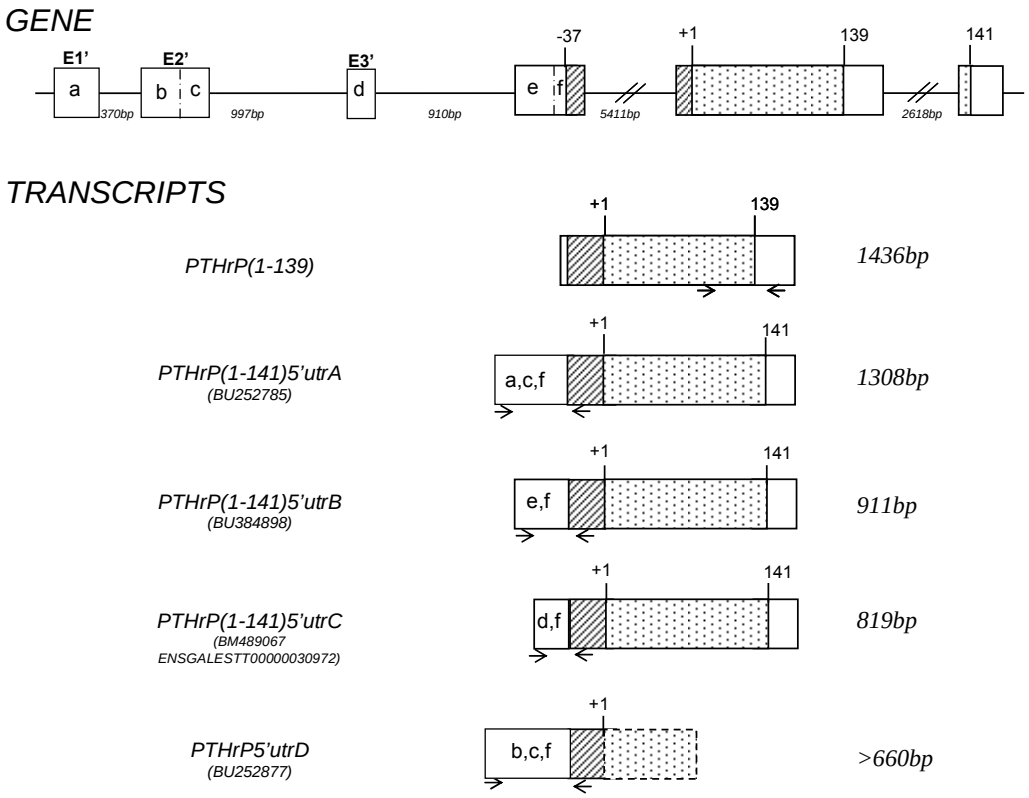


Figure 16: Chicken PTHrP alternative transcripts. The five PTHrP transcripts that result from alternative exon skipping events are mapped against the structure of the chicken PTHrP gene and their respective EST accession number and size (bp) indicated. The novel chicken transcripts were named according to the size of the deduced mature peptide (139 or 141) precursor and the length of their 5'UTR region (A to D). Arrows delimit regions amplified by q-PCR for each transcript and the deduced mature peptide sequence of each transcript is given and +1 indicates the start of the mature peptide. Coding exons are represented by filled boxes, non-coding exons by open boxes and introns by lines and the dotted-filled boxes indicate the mature PTHrP peptide region. Non-coding 5'UTR exons are designated by E1' to E3' and the predicted intron sizes (bp) of the chicken PTHrP gene are given. For simplicity, the 5'UTR regions transcribed from non-coding exons are designated by letters (a to f) and the dashed/dotted line within the E2' and E1 region indicate the alternative splicing events. PTHrP 5'utrD EST was found to be incomplete and only part of the mature PTHrP peptide was characterized.

Transcripts – PTH-L

The gene structure of *Xenopus PTH-L* has only been partly elucidated and was composed of at least two exons and appears to have an identical gene structure to PTH (Figure 15). The deduced protein sequence of PTH-L (accession number FM955443) is 152 amino acids (Figure 13).

Two main transcripts for Xenopus PTH-L (XenPTH-L) were identified by clustering seven XenPTH-L ESTs (Table 2). One transcript (XenPTH-L*5utrE1'*) was represented by three ESTs from gastrula stages 10.5-12 (AL964863) and a mixture of brain and spinal

cord from tadpoles stages 58-64 (CN076481; CN076482). The second transcript (XenPTH-L*5utrE2'*) was represented by four ESTs from gastrula stages 10.5-12 (AL775245; AL965929; BX750398; BX764109). The deduced XenPTH-L was 118 amino acids long and yielded a mature protein of 86 amino acids (Figure 15 and Figure 11). The chicken PTH-L gene had a similar structure to the PTH gene and was composed of two exons and one intron. No ESTs for ckPTH-L were identified although RT-PCR indicated it is expressed in several tissues (Figure 17).

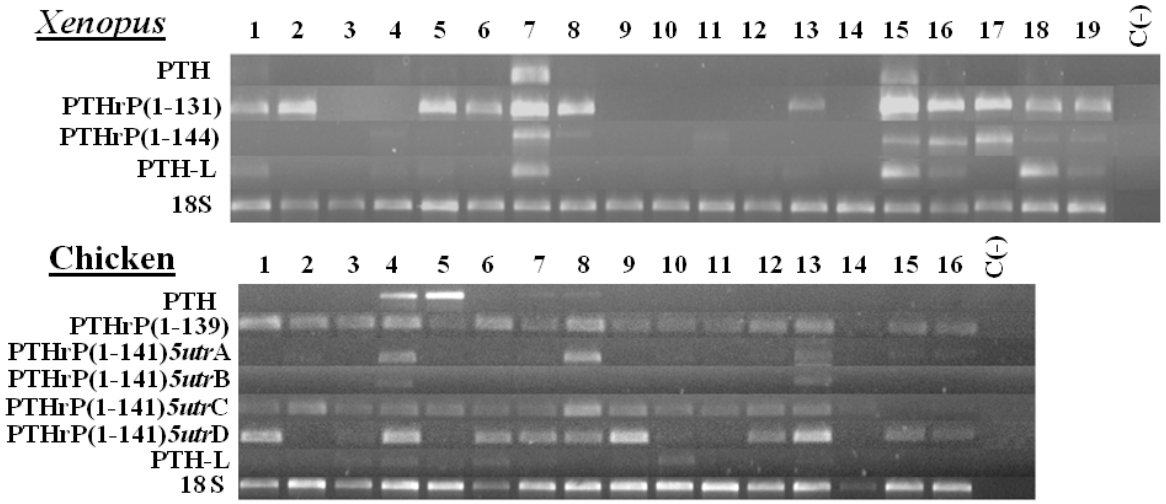


Figure 17: RT-PCR expression of PTH/PTHrP family members in adults of *Xenopus* (A) and chicken (B). Gene specific primers were designed in order to amplify the *Xenopus* and chicken PTH and PTH-L transcripts and the PTHrP isoforms. In (A) the adult *Xenopus* tissues analyzed were spleen (1), skin (2), muscle (3), cartilage (4), bone (5), kidney (6), gall bladder (7), esophagus (8), stomach (9), duodenum (10), hindgut (11), midgut (12), liver (13), brain (14), lung (15), heart (16), gonads (17) and thyroid (18 and 19). In (B) the adult chicken tissues analyzed were forebrain (1), midbrain (2), hindbrain (3), pituitary (4), parathyroid (5), thyroid (6), bone (7), cartilage (8), muscle (9), kidney (10), liver (11), lung (12), eggs (13), duodenum (14), hindgut (15) and midgut (16). C $^{(-)}$ represents the negative control reaction. The ribosomal unit 18S was used as an internal control to normalize RT-PCR reactions and amplified products were sequenced to confirm identity.

2.3.3. Phylogenetic analysis

The topologies of phylogenetic trees constructed with Neighbor Joining or parsimony methods were similar and a consensus tree is presented (Figure 18). PTH, PTHrP and PTH-L were separated into different clades and the *Xenopus* and chicken PTH-like family members clustered with their respective vertebrate homologues (Figure 18).

In teleosts, a specific duplication of the PTH and PTHrP genes occurred although only one PTH-L exists and this may be a consequence of; 1) deletion of the second copy

from teleost genomes or 2) a partial duplication of teleost PTH-like members. The isolation of homologues of *Takifugu* PTH-L gene in amphibian and chicken genomes suggests that members of the PTH/PTHrP family emerged prior to the teleost divergence and were subsequently maintained in vertebrate genomes. The exception is eutherian mammals which lack a PTH-L gene.

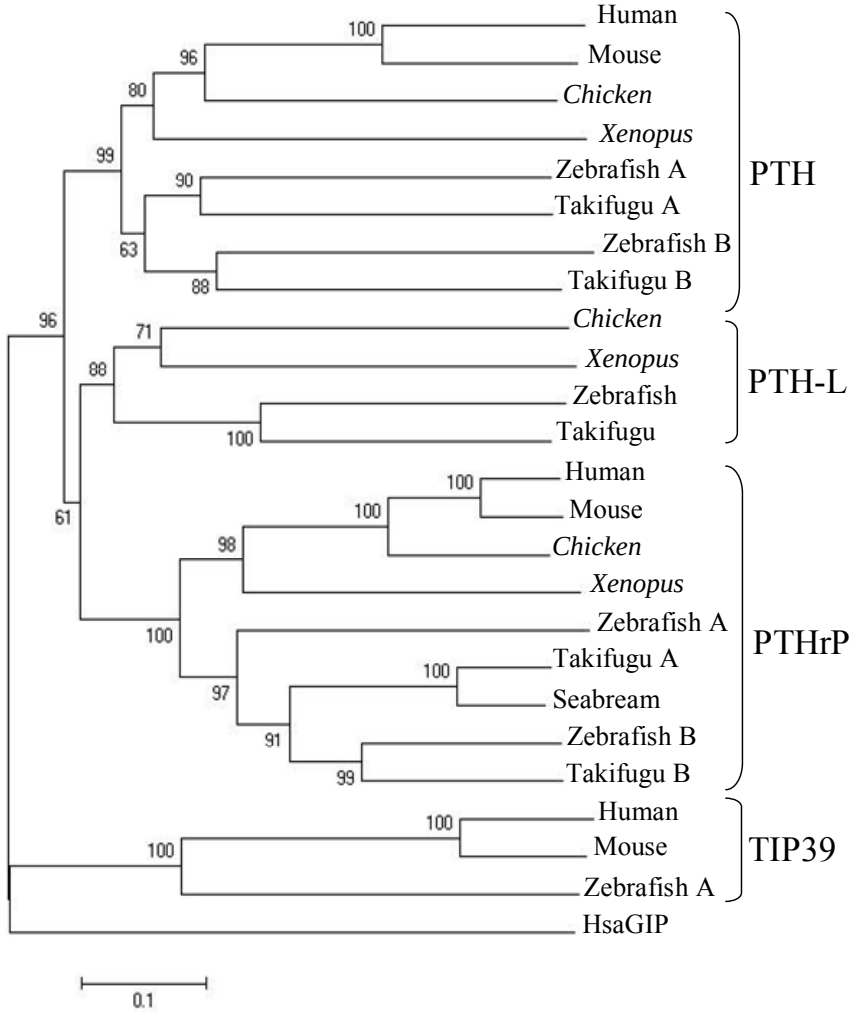


Figure 18: Consensus phylogenetic tree of *Xenopus* and chicken PTH-family members using the Neighbor Joining method (Saitou and Nei 1987) and 1000 bootstraps replicates with the complete amino acid precursor sequence in Mega3.1 software (Kumar, Tamura et al. 2004) with the settings pairwise deletion, p-distance model and 222 informative sites. *Xenopus* and chicken PTH-family members are in italics and the sequence of human GIP (HsaGIP, NP_004114) was used as outgroup. Human (NP_848544), mouse (NP_444486) and zebrafish (NP_991140) TIP39 mature protein sequences were included for comparative purposes. The accession numbers of other sequences utilized for tree construction are indicated in Figure 14 and seabream PTHrP is AAF79073.

2.3.4. Short-range gene environment comparisons

To better understand the evolution of vertebrate PTH-family members, the gene environment of the *Xenopus* and chicken genes were characterized and compared with the homologue regions in *Takifugu* and human. Short-range comparisons indicate that gene synteny and gene order were maintained across vertebrates suggesting that members of this family evolved under conservative pressures (Figure 19).

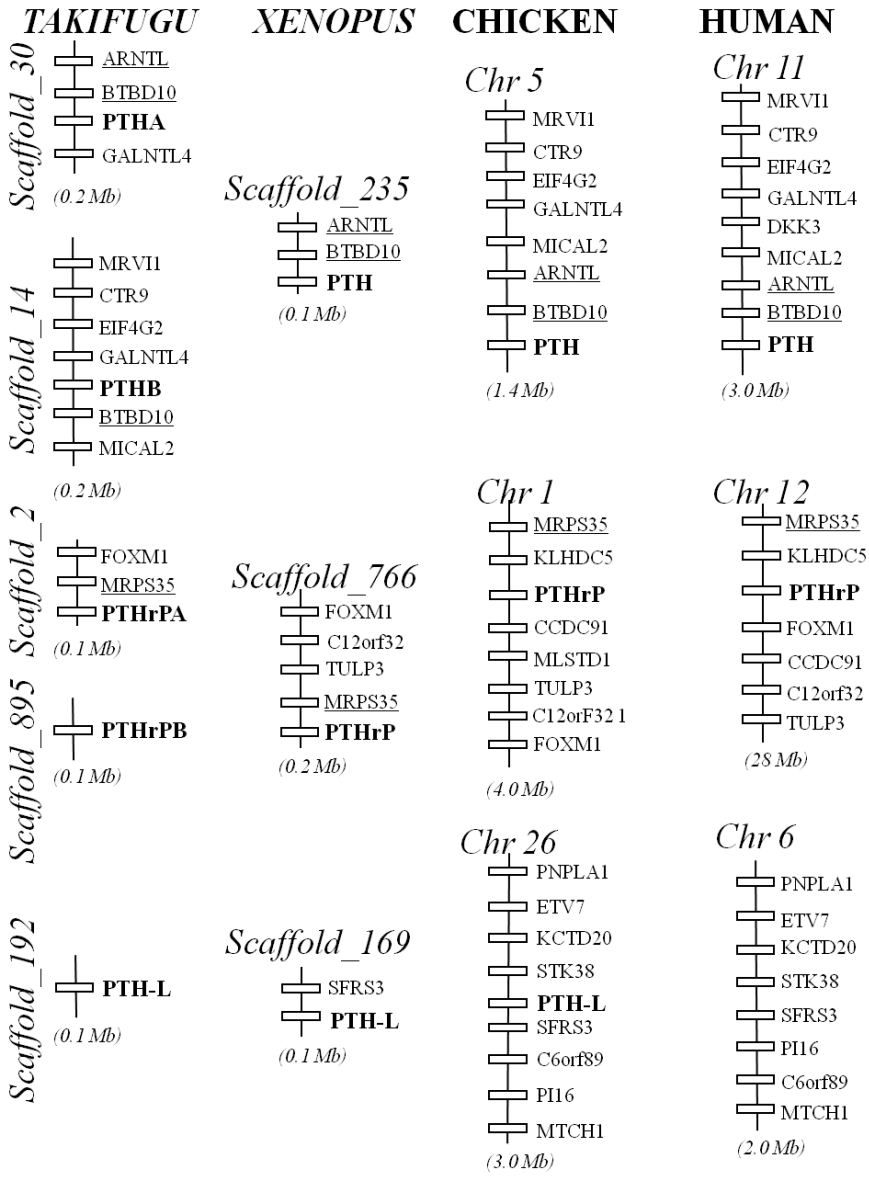


Figure 19: Short-range gene linkage comparisons of the PTH-family members in the *Takifugu*, *Xenopus*, chicken and human genomes. Genes are represented by closed boxes and the size of the chromosome region analysed is given underneath. Genes were named using HUGO and lines indicate chromosome/scaffold segments. The vertebrate PTH-family members are in bold and conserved flanking genes identified within the homologue regions are underlined. The PTH gene is localized in *Xenopus* scaffold_235 and in chicken chromosome 5 and two conserved genes ARNTL and BTBD10 were identified. The *Xenopus* and chicken PTHrP maps to scaffold_766 and

chromosome 1, respectively and the gene MRPS35 was found in close proximity in all vertebrate regions analysed. PTH-L and SFRS3 genes map to *Xenopus* scaffold_169 and to chicken chromosome 26. SFRS3 was not linked to *Takifugu* PTH-L and is present on human chromosome 6 which lacks PTH-L. For simplicity, only genes with correspondence across species are represented. The figure is not drawn to scale.

The chicken and human homologue genome segments were the most highly conserved and similar linked genes were identified flanking PTH-family members. The genes *ARNTL* and *BTBD10* were localized in close proximity to *PTH*. Genes *MRPS35* and *SFRS3* were identified within the *PTHrP* and *PTH-L* homologue regions, respectively. The genes flanking *PTH-L* in *Xenopus* and chicken were identified on human chromosome 6, although *PTH-L* was lacking suggesting that specific gene/genome rearrangement events occurred during the mammalian radiation.

2.3.5. PTH-family genes expression

The tissue distribution and relative abundance of all *Xenopus* and chicken PTH-family transcripts was investigated by RT-PCR (Figure 17) and q-PCR (Figure 20). PTH and PTHrP transcripts had a similar tissue distribution in both *Xenopus* and chicken.

PTH-L had a widespread tissue distribution in *Xenopus* and the highest transcript abundance (copy number relative to 18S) was detected in brain, lung and bone (Figure 20). *Xenopus* PTHrP transcripts were detected in brain, lung, skin and heart and were low abundance in spleen and muscle (Figure 20). It was not possible to design q-PCR primers which discriminated between the two *Xenopus PTHrP* transcripts. However, RT-PCR (Figure 17) revealed both transcripts have a similar distribution. *Xenopus* PTH was expressed in bone, kidney, lung and nervous tissue (mixed brain and pituitary). The presence of PTH-family members was not established in the amphibian PTG as its small size and variable morphology made it difficult to collect (Srivastav, Das et al. 1995).

In chicken, *PTH* transcripts were highly abundant in the PTG (Figure 20), although they were also detected at low abundance in the pituitary, thyroid, bone and cartilage. Chicken PTHrP transcripts were widely expressed (Figure 20 and Figure 17) and the various isoforms had a differential tissue distribution suggesting they may have different functional roles. ckPTHrP(1-141)*5utrB* and ckPTHrP(1-141)*5utrC* were the most abundant transcripts (copy number relative to 18S) and were mainly found in cartilage. ckPTHrP(1-141)*5utrB,* ckPTHrP(1-141)*5utrC* and ckPTHrP(1-141)*5utrD* transcripts had a similar tissue distribution. The tissue distribution of ckPTHrP(1-139) overlapped with ckPTHrP(1-141)*5utrA* with the exception of cartilage in which the latter transcript was absent. ckPTHrP(1-139) was highly expressed in muscle and was the only isoform which was not

detected in the PTG. It was also absent from bone together with ckPTHrP(1-141)*5utrA*. In contrast, both isoforms were expressed in kidney where ckPTHrP(1-141)*5utrB* and PTHrP(1-141)*5utrC* were not detected. ckPTH-L transcripts had a similar tissue distribution to ckPTHrP (Figure 20) and were most abundant in cartilage but were also present in pituitary, thyroid, PTGs, muscle, hind brain and kidney.

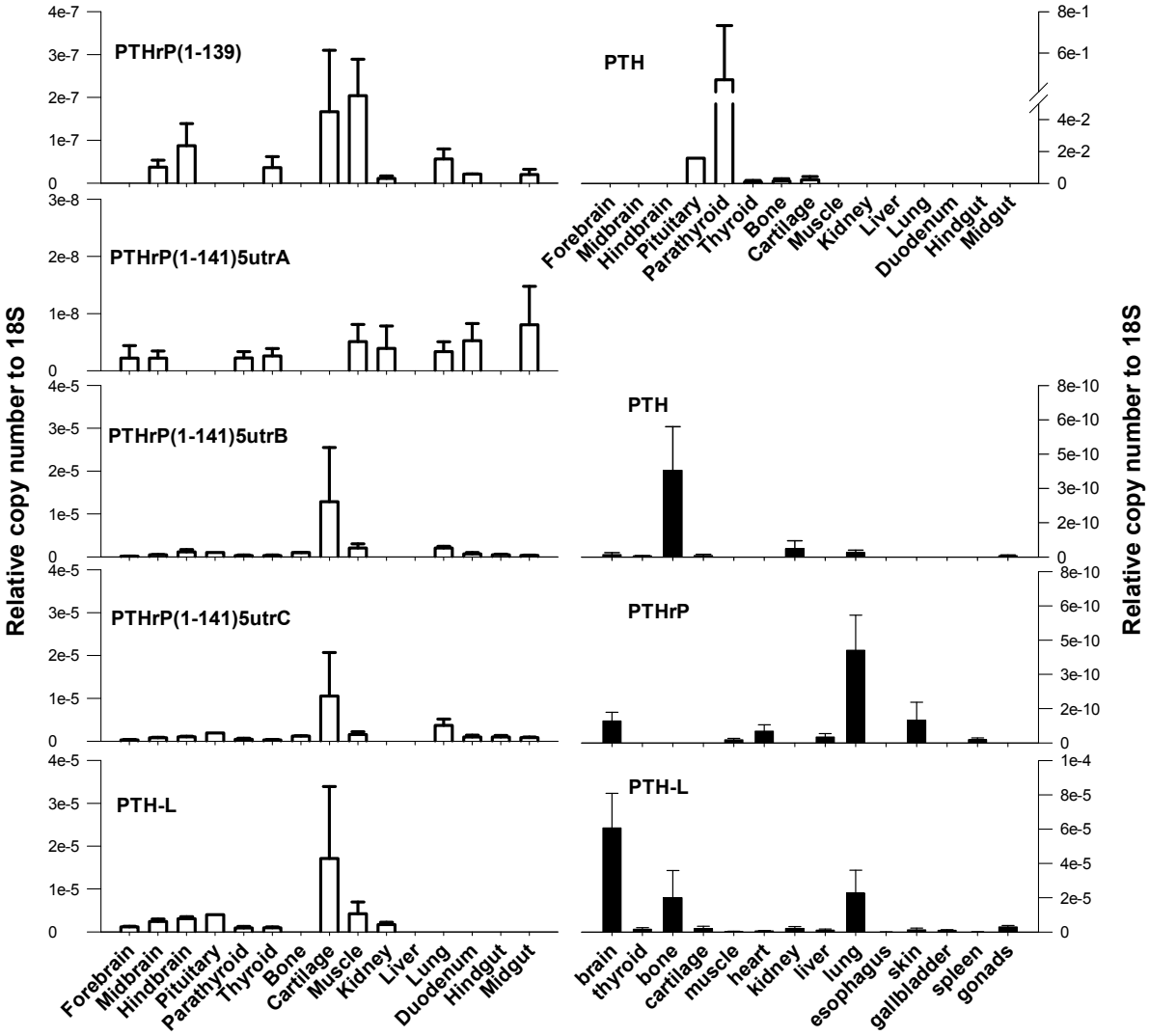


Figure 20: Expression of chicken (open bars) and *Xenopus* (closed bars) PTH-family genes as determined by q-PCR. Gene specific primers were used to amplify PTH and PTH-L transcripts and PTHrP spliced isoforms from several tissues. The number of amplified transcripts is presented in relation to 18S copy number and data is presented as mean ±S.E. (n=2 to 3 for *Xenopus* and n=3 for chicken except for pituitary where n=1).

2.3.6. Transepithelial calcium transport

The resistance and short-circuit current of *Xenopus* abdominal skin and chicken CAM membranes were substantially different (Table 3). CAM is a leaky epithelium and the frog skin is a tight epithelium and both have calcium transporting capacity. All *Xenopus* and chicken (1-34) N-terminal peptides were able to increase unidirectional calcium fluxes from the apical/basolateral membrane site (Figure 21). XenPTH(1-34) changed calcium fluxes across *Xenopus* abdominal skin from 2.6 $nmol.h^{-1}.cm^2$ to 6.1 $nmol.h^{-1}.cm^2$, XenPTHrP (1-34) from 3.8 $nmol.h^{-1}.cm^2$ to 11.6 $nmol.h^{-1}.cm^2$ and XenPTH-L(1-34) from 1.4 $nmol.h^{-1}.cm^2$ to 5.2 $nmol.h^{-1}.cm^2$.

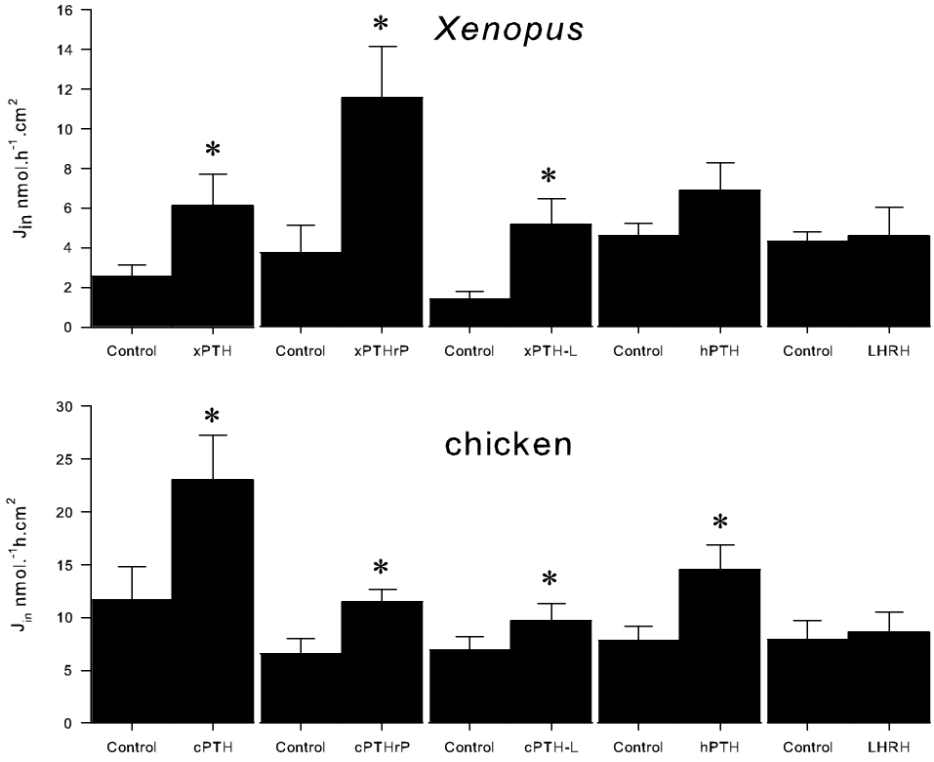


Figure 21: Calcium fluxes (water to blood side) in *Xenopus* abdominal skin and chicken 16 to 18 day old embryo CAM (shell to embryo side) after the addition of 10nM, N-terminal (1-34) PTH, PTHrP and PTH-L to the basolateral membrane site. Human PTH (1-34) and salmon Luteinizing hormone-releasing hormone (LHRH) were used as positive and negative controls respectively. Results are shown as mean ± SEM and the "*" indicates statistical significance compared to control (time 0) ($p<0.05$).

A significant increase in short-circuit current (Isc) was observed after 50 and 60 minutes of exposure of Xenopus abdominal skin to XenPTHrP(1-34) and XenPTH-L(1-34), respectively which suggests these peptides may also be involved in the transport of other ions in the skin (Table 3). This possibility was tested by comparing theoretical and

measured short-circuit current using the formula *Isc* = *Ji.z.F* (where *Ji*, represents the uptake, *z* the calcium valence and *F* is the Faraday constant) (Koefoed-Johnsen and Ussing 1958). For PTHrP the calculated value for *Isc* (in μ/cm^2) was 0.84 vs. the measured value 0.32. For PTH-L the calculated value for *Isc* was 0.41 vs. the measured value 0.24. The values of *Isc* for the two peptides are within the same range. The difference between calculated and measured values of *Isc* is indicative of an additional transport mechanism responsive to both peptides, e.g. secretion of anions (likely chloride).

CkPTH(1-34) doubled calcium flux through CAM from 12.5 $nmol.h^{-1}.cm^2$ to 23.5 $nmol.h^{-1}.cm^2$, ckPTHrP (1-34) from 6.5 $nmol.h^{-1}.cm^2$ to 11.5 $nmol.h^{-1}.cm^2$ and ckPTH-L(1-34) from 6.8 $nmol.h^{-1}.cm^2$ to 9.8 $nmol.h^{-1}.cm^2$ (Figure 21). Human PTH(1-34) induced a significant increase in transepithelial calcium transport in chicken CAM but had no effect on *Xenopus* abdominal skin (Figure 21). Salmon LHRH was used as a negative control and had no effect on calcium transport in either skin or CAM confirming the specificity of the effects observed with the chicken and *Xenopus* peptides.

Table 3: Variation in bioelectric values of *Xenopus* abdominal skin and chicken CAM measured *in vitro* prior and at different times after the basolateral application of 10nM of *Xenopus* or chicken PTH(1-34) family peptides. Note: Isc (μAmp/cm^2); Rt (Ω.cm^2); * indicates statistically significant difference from time=0 control ($p<0.05$)

		Time after hormone application (min)						
Xenopus		**0**	**10**	**20**	**30**	**40**	**50**	**60**
PTH (n=11)	Isc	2.49±0.34	2.45±0.33	2.43±0.32	2.44±0.30	2.48±0.29	2.48±0.30	2.49±0.28
	Rt	1143±128	1157±129	1164±129	1130±126	1160±127	1164±128	1167±27
PTHrP (n=12)	Isc	2.59±0.31	2.63±0.30	2.67±0.29	2.74±0.29	2.82±0.28	*2.88±0.27	*2.91±0.25
	Rt	1329±140	1320±122	1328±122	1276±104	1273±98	1263±95	1264±95
PTH-L (n=12)	Isc	2.36±0.42	2.36±0.43	2.39±0.42	2.44±0.42	2.51±0.42	2.56±0.43	*2.59±0.43
	Rt	1332±81	1328±75	1335±79	1318±79	1327±78	1322±76	1335±76
Chicken								
PTH (n=11)	Isc	7.41±1.90	7.54±1.84	7.39±1.86	7.33±1.88	7.02±2.00	7.14±2.06	7.03±2.08
	Rt	190±20	191±20	194±21	196±21	191±23	194±23	196±24
PTHrP (n=9)	Isc	12.53±2.77	13.09±2.76	13.02±2.74	13.05±2.73	13.03±2.69	13.22±2.76	13.06±2.73
	Rt	181±15	182±15	181±15	179±15	174±15	176±15	177±16
PTH-L (n=12)	Isc	7.22±1.26	7.37±1.18	7.15±1.18	6.86±1.10	6.75±1.08	6.80±1.09	6.66±1.10
	Rt	211±21	208±20	211±20	207±21	203±20	205±20	207±30

2.4. Discussion

In the current study, three PTH-family members, *PTH*, *PTHrP* and *PTH-L*, were characterized in *Xenopus* and chicken. Preliminary functional analysis suggests the action on calcium uptake of the (1-34) N-terminal region is conserved in PTH-family members. Comparison of sequence, structure and gene environment suggests they have evolved by gene duplication and deletion events. Moreover, a number of novel *PTHrP* splice variants with a variable 5'UTR were also identified. All three PTH-family members are present in teleost and tetrapod genomes with the exception of eutherian mammals which have lost the PTH-L gene, probably because of specific gene or genome rearrangements during the mammalian radiation. This hypothesis is supported by the conservation in human chromosome 6 of genes linked to *PTH-L* in fish, *Xenopus* and chicken. Phylogenetic analysis supports previous theories that PTH-like family members evolved from a common ancestral precursor, from which tuberoinfundibular peptide 39 (TIP39) has also been suggested to have arisen (John, Arai et al. 2002). To date, PTH-family homologues remain to be identified in invertebrates and their emergence in vertebrates coupled with their important role in calcium homeostasis and skeletal development suggest that their origin may be associated with the acquisition of a calcified endoskeleton.

Analysis of PTH-family members in *Xenopus* and chicken indicates that similar post-translation modifications to those previously described for the human homologue occur (Habener, Kemper et al. 1976; Orloff, Reddy et al. 1994). The bioactive N-terminal (1-34) peptide of PTH-family members is the most conserved region of the protein (Figure 14). The physicochemical properties of amino acids in the N-terminal region including L^{24} and L^{28} which are important for receptor activation (Jin, Zhang et al. 1991; Murray, Rao et al. 2005), are maintained in *Xenopus* and chicken suggesting they may have similar functional roles across the vertebrates. Moreover, within this region the highest conservation is found in the first residues. Previous studies comparing activation of teleost and human PTHR by PTHrP and PTH suggests they have distinct activation profiles and indicate the C-terminal region of the protein is also involved (Rubin, Hellman et al. 1999; Shimizu, Potts et al. 2000; Rotllant, Redruello et al. 2005).

In addition to amino acid sequence conservation, the gene organization of *Xenopus* and chicken PTH and PTH-L is also maintained (Figure 12) (Ingleton and Danks 1996; Power, Ingleton et al. 2000). However, although PTH-L splice isoforms have been found in *Xenopus* and *Takifugu* (Canario, Rotllant et al. 2006) no alternative PTH splice isoforms have been identified in any species to date. In contrast, the gene structure of PTHrP is poorly conserved between vertebrates and unique *Xenopus* and chicken isoforms which result from exon skipping events, were identified and resemble those

reported for the human gene (Philbrick, Wysolmersky et al. 1996). In human, three PTHrP transcripts are expressed as a result of alternative promoter usage, two of which (139 and 141 deduced amino acids) are identical in size to those present in chicken. The only difference between the mature proteins in both human and chicken is the presence in the 1-141 *PTHrP* isoform of two extra C-terminal amino acids (Figure 13). In *Xenopus* a unique *PTHrP* splice isoform results from a splice event within the osteostatin region and the incorporation of a novel coding sequence and 3'UTR giving rise to a longer peptide precursor (144 amino acids). In contrast, to terrestrial vertebrates including *Xenopus* where splice variants of PTH-family members are common, none were identified in teleosts. Variation in the UTR has been associated with tissue-specific (Mignone, Gissi et al. 2002), developmental stage or cell state specific (Kreth, Ledderose et al. 2008) regulatory mechanisms. The existence of splice variants suggests that complex regulatory mechanisms for this gene family were acquired within the tetrapod radiation and this may be related to the change in mineral homeostasis which accompanied the adaptation of vertebrates to a terrestrial environment (Ingleton and Danks 1996; Abbink and Flik 2006; Guerreiro, Renfro et al. 2007).

The presence of distinct *Xenopus* and chicken *PTHrP* isoforms and their widespread distribution and expression in bone, cartilage, skin and kidney (classical tissues involved in calcium regulation) further supports their role as important calciotropic factors. The PTHrP transcripts identified in chicken are predicted to produce mature proteins with the same length as the human PTHrP(1-139) and PTHrP(1-141) proteins, although no homologue of the human PTHrP(1-173) was identified suggesting it may be a human innovation. The *Xenopus* and chicken PTHrP and PTH-L tissue expression overlaps in the majority of the tissues analysed suggesting widespread paracrine actions for the two proteins.

PTH distribution is more restricted and in chicken, as expected, is expressed abundantly and almost exclusively in the PTG (Figure 20). In *Xenopus*, we were not able to identify the parathyroid tissue and it has previously been noted to be inconspicuous and readily degenerates (Cortelyou and McWhinnie 1967; Srivastav, Das et al. 1995). In chicken PTH expression was also detected in the pituitary gland and in *Xenopus* brain/pituitary extracts, which is in agreement with studies which showed that immunoactive protein was detected in pituitaries from sheep (Balabanova, King et al. 1985), and fish (Pang, Kaneko et al. 1988; Guerreiro, Renfro et al. 2007) (which lack an organized PTG structure). PTH immunoreactivity in brain and pituitary was also reported for several tetrapods, including chicken (Pang, Harvey et al. 1988), but so far has only been confirmed in rabbit and rat brain/pituitary. Taken together these results suggest a high degree of conservation of pituitary PTH expression and possibly secretion across

vertebrates, although in mouse the thymus is also a source of PTH (Günther, Chen et al. 2000). *PTH* expression in *Xenopus* lung has not, to our knowledge, been previously reported. However, the overall PTH tissue distribution largely coincides with the two PTH/PTHrP receptors identified in this organism (Bergwitz, Klein et al. 1998). This supports the hypothesis that PTH may have a more paracrine action in lower vertebrates.

Although there was an overlapping tissue distribution of some of the transcripts of the three PTH-family members, expression of *PTH* and *PTH-L*, unlike *PTHrP*, is more restricted (Figure 20). The *PTHrP* isoforms identified in chicken seem to conform to specific patterns and levels of abundance, although only one of the transcripts produces a different protein. With the exception of chicken PTHrP(1-141)*5utrA*, the other chicken PTHrP are expressed in cartilage at high levels confirming their important role in this tissue (Farquharson, Jefferies et al. 2001). However, in *Xenopus PTHrP* transcript seemed to be absent from cartilage. The different tissue expression profiles of PTH-family members in *Xenopus* and chicken [and *Takifugu* (Canario, Rotllant et al. 2006)] suggest that specific modulation of gene expression occurs and detailed analysis of the promoter region may help clarify this question.

All *Xenopus* and chicken PTH-like peptides were found to promote unidirectional calcium influx (Figure 21). The differing effect of human PTH(1-34) in chicken and *Xenopus* is in keeping with what has been previously observed for the bovine peptide (Schermer, Bradley et al. 1994; Stiffler, Yee et al. 1998). However, after pre-treatment with Vitamin D, bovine PTH(1-34) is also able to stimulate calcium transport across the *Rana pipiens* skin epithelium (Stiffler, Yee et al. 1998). *Xenopus* PTH-L and chicken PTH peptides were the most effective in promoting calcium transport across the membranes in each specific assay. Similar results were obtained for transport across teleost larvae epithelia (Canario, Rotllant et al. 2006) and although more studies are required to establish their specific function, the results provide new insight into the interlink between evolution and function of the PTH-family.

Although the three peptides enhance calcium transport, only PTHrP and PTH-L caused changes in the short-circuit current in *Xenopus* skin, indicating an additional effect on transport of ions other than calcium (Table 3). Similar effects on the short-circuit current in the same range of PTH concentrations have previously been observed with A6 cells derived from *Xenopus* kidney (Rodriguez-Commes, Forrest et al. 1995); on sodium transport of PT cells derived from chicken kidney (Laverty, McWilliams et al. 2003); and with PTHrP on fish intestine (Fuentes, Figueiredo et al. 2006). Our results further substantiate the effect of the PTH-family of proteins on the short-circuit current and suggest that the action of the different peptides on the short-circuit current is tissue dependent and may vary with species.

While *PTHrP* and *PTH* genes have been identified in teleosts and tetrapods, *PTH-L* seems to be absent from eutherian mammals and it was recently suggested to be the functional homologue of mammalian *PTH*, in fish (Canario, Rotllant et al. 2006). In the present study, *Xenopus* PTH-L in common with teleost PTH-L, was the most potent peptide promoting calcium transport across epithelia. In chicken, a different scenario was observed and PTH seemed to be more efficient in stimulating calcium transport. Taking into consideration a) the reduced calciotropic activity of PTH-L in chicken, b) the overlapping distribution between PTH-L and PTHrP and c) the absence of a PTH-L in the human genome (data not shown), it is proposed that a transitory functional role between the vertebrate PTH-L and the tetrapod PTHrP and PTH occurred after the amphibian divergence. The tetrapod PTH-L probably acquired a minor role and became potentially non-functional and was consequently eliminated from the human genome. In contrast, PTH which in teleosts and *Xenopus* appears to be a paracrine factor seems to have gained a more important functional role during evolution and became a major endocrine factor with a restricted production in a highly specialized structure, the PTG (Okabe and Graham 2004). Future studies using more in depth functional studies and other organisms will be needed to support this hypothesis.

As conclusion, the parathyroid hormone family contains 3 principal members, *PTH*, *PTHrP* and the recently identified *PTH-L*. In teleosts there are 5 genes which encode *PTHrP* (2), *PTH* (2) and *PTH-L* and in tetrapods there are 3 genes (*PTHrP*, *PTH* and *PTH-L*), the exception is placental mammals which have 2 genes and lack a *PTH-L*. Gene structure of *PTH* and *PTH-L* seems to be conserved throughout the vertebrates while PTHrP gene structure is divergent and it has acquired new exons and alternative promoters. The highly conserved *Xenopus* and chicken N-terminal (1-34) region of PTH, PTHrP and PTH-L has the capacity to stimulate calcium uptake across, respectively, the frog skin and chicken chorionallantois membranes, indicating a conserved role in calcium metabolism possibly via similar receptors. It is hypothesized that PTH-family genes appeared at approximately the same time during the vertebrate radiation and evolved via gene duplication/deletion events. During evolution PTH-L was lost from the genome of eutherian mammals, while PTH, which has a paracrine distribution in lower vertebrates, became the product of a specific endocrine tissue, the PTG and gained an important role in calcium regulation. The PTHrP gene acquired during its evolution a disparate and more complex organization in vertebrates probably associated with its paracrine nature and pluripotent functions.

CHAPTER III

Functional Characterisation of the Chicken PTH/PTHrP Receptors: Evidence for Loss of the PTH2R Homologue in the Avian Lineage

With: João C.R. Cardoso, Deborah M. Power and Adelino V.M. Canário.

Abstract

The parathyroid hormone (PTH)-family members are important calciotropic peptides involved in the regulation of calcium homeostasis in vertebrates. They interact with specific members of family 2 GPCRs which have been characterised in teleost and mammals. Two PTH receptors, PTH1R and PTH2R are present in mammals and in teleost fish, the latter also with a third receptor PTH3R. The existence of specific chicken PTH-family receptors has previously reported, however they remain to be isolated and functionally characterized. The aim of this study is to go further in the understanding of the function and evolution of the chicken PTH/PTHrP endocrine system by the isolation and functional characterisation of their specific receptors.

Two PTHRs were identified in chicken, homologues of PTH1R and PTH3R. The two PTHRs contain the conserved structural and functional motifs characteristic of other vertebrate PTHRs and share a similar gene structure organisation with their teleost fish homologues. There is conservation of gene synteny and gene order with the receptors in other vertebrates. Phylogenetic analysis suggests that the chicken receptors are duplicates and that they emerged prior to teleost/tetrapod divergence.

PTH1R and PTH3R have a widespread gene expression in different tissues and developmental stages of chicken. In cell lines stably expressing the two receptors, the chicken PTH, PTHrP and PTH-L peptides were able to elicit cAMP accumulation in a dose-dependent manner, with PTHrP as the most effective. PTHrP was also able to stimulate PTH1R intracellular Ca^{2+} accumulation suggesting that it can activate alternative signalling pathways within the same receptor. This plus the fact that both receptors can be co-expressed highlights the complexity of the mechanism of action of the PTH-system and its multiple roles in vertebrates.

3.1. Introduction

The parathyroid hormone (PTH) system is the main endocrine mechanism involved in the maintenance of calcium homeostasis, proposed to have emerged with the acquisition of the vertebrate skeleton. Owing to its biomedical importance the PTH system has been mainly studied in mammals and recently in some fishes. The PTH system is composed of three peptide hormones, which share a conserved 1-34 amino acid N-terminal region involved in receptor binding and activation. In mammals, the PTH and PTH-related protein (PTHrP) have been characterised (Keutmann, Sauer et al. 1978; Moseley, Kubota et al. 1987) and in fish, duplicated homologues of the mammalian forms (PTH1/PTH2 and PTHrPA/PTHrPB) (Ingleton 2002; Guerreiro, Renfro et al. 2007) and a PTH-Like peptide (PTH-L) were isolated (Canario, Rotllant et al. 2006). Two specific receptors (PTH1R and PTH2R) have been characterised in mammals and in zebrafish, in addition to PTH1R and PTH2R homologues, a third receptor, PTH3R, is also present. The identification of duplicates of the mammalian homologues in teleosts is proposed to be a consequence of their specific genome duplication event (Juppner, Abou-Samra et al. 1991; Rubin and Juppner 1999). PTHRs are members of the family 2 B1 G-Protein Coupled Receptors (GPCRs) (Gensure, Gardella et al. 2005) which is a large group of seven transmembrane peptide and neuro-endocrine receptors identified in vertebrates (Harmar 2001; Cardoso, Pinto et al. 2006). Receptors of this family are also characterised by the presence of large N-terminal extracellular region involved in ligand interaction and by a C-terminal intracellular domain that is responsible for the activation of the intracellular signalling cascade (Juppner, Abou-Samra et al. 1991; Gardella and Juppner 2001; Harmar 2001). At the receptor N-terminal region, six conserved cysteine residues and N-glycosylation sites are responsible for the formation of the ligand-binding pocket. Receptor activation trigger different intracellular signalling pathways, including the activation of protein kinases A (PKA) and accumulation of cAMP (Juppner, Abou-Samra et al. 1991; Li, Dong et al. 2004) and phospholipase C leading to protein kinase C (PKC) inducing intracellular Ca^{2+} release (Zuscik, O'Keefe et al. 2002).

Studies using *in vitro* cell assays suggest that the mammalian and teleost ligands are functionally distinct. For example, the zebrafish PTH2R binds exclusively to tuberoinfundibular peptide 39 (TIP39), a peptide that shares similar secondary structure to PTH(1-34) while the mammalian receptor homologue is also activated by PTH. However, fish PTHrPA and mammalian PTHrP bind preferentially to PTH1R and PTH3R (Usdin, Hoare et al. 1999; Papasani, Gensure et al. 2004; Gensure and Juppner 2005). A specific receptor for the fish PTHrPB and PTH-L remains to be assigned.

Recently, homologues of the mammalian *PTH* and *PTHrP* and teleost *PTH-L* were identified in chicken and *Xenopus*, and preliminary studies indicate that they have a conserved action in calcium *transport* (Pinheiro, Cardoso et al. 2010). Moreover, as with teleost fish and mammals, PTHrP was found to be the most widespread transcript and distinct chicken *PTHrP* isoforms similar to those reported from human were found. Furthermore, *PTH* was found to be highly expressed in the chicken PTG and to be the most potent peptide involved in calcium transport.

A potential PTH1R activated by PTHrP was suggested to be expressed in chick bone and kidney (Forte, Langeluttig et al. 1982; Pliam, Nyiredy et al. 1982; Zhao, Brauer et al. 2002). In addition it has also been suggested that tibial growth plate chondrocytes proliferation is promoted by PTHrP and evokes PKA activation (Zuscik, Puzas et al. 1994) and that PTH involvement in proteoglycan synthesis is Ca^{2+} dependent (Zuscik, O'Keefe et al. 2002). However, PTH receptors remain to be isolated in chicken (Lagerstrom, Hellstrom et al. 2006).

The aim of the present study is to isolate and functionally characterise the chicken PTHRs. For that purpose, putative chicken PTHRs were identified using *in silico* analysis of the chicken genome and expressed sequence tags (EST) from public databases. Gene expression in chicken adult and several embryonic developmental stages were characterised and the relative potency of chicken PTH members 1-34 N-terminal peptides and human PTH and TIP39 in stimulating receptor activity was measured by the quantification of intracellular cAMP production and Ca^{2+} accumulation.

3.2. Methods

3.2.1. Animals and tissue collection

Adult white leghorn chickens (*Gallus gallus*) were supplied by a local farm and were anesthetized with diethyl ether (Merck, Spain) before sacrificed by decapitation. Fertile chicken eggs were obtained from Quinta da Freiria (Serpa, Portugal) and kept at 37.6º C under high humidity conditions in an automatic incubator (Brinseca OCTAGON 40) with gentle rotation. Several tissues were collected from adults and during selected chick embryonic developmental stages: 4HH (definitive primitive streak process); 6HH (head and neural folds); 17HH (leg bud formation); 26HH (toes formation); 31HH (feather germs; emergence of the interdigital membrane) and 36HH (labial groove; uropygial gland) (Hamburger and Hamilton 1992). The chorionallantois membrane (CAM) from 44HH that was previously demonstrated to be involved in calcium transport (Pinheiro, Cardoso et al. 2010) was also collected. Tissues were snap frozen in liquid nitrogen and

stored at -80ºC until use. All procedures with animals were performed in accordance with Portuguese legislation under a “Group-1” licence from the Direcção-Geral de Veterinária, Ministério da Agricultura, do Desenvolvimento Rural e das Pescas, Portugal.

3.2.2. Sequence database searches

The chicken (*Gallus gallus*) genome and EST databases were searched using the human (PTH1R, AAR18076; PTH2R, AAH36811) and zebrafish (PTH1R, NP_571432; PTH2R, NP_571452; PTH3R, NP_571453) deduced protein receptor sequences with the TBLASTN and default settings (Altschul, Gish et al. 1990) against the Ensembl genome assembly (http://www.ensembl.org, accessed at 7/2007) and NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/). Chicken ESTs were retrieved from Biotechnology and Biological Sciences Research Council (BBSRC:,http://www.chick.manchester.ac.uk/) and also from the NCBI A*ves* dbEST subset (taxid:8782) using a similar strategy.

Searches were also performed against genome and available nucleotide and protein data from other non-mammalian tetrapods deposited in Ensembl for the birds zebra finch (*Taeniopygia guttata*) and turkey (*Meleagris gallopavo*), amphibian *Xenopus (Xenopus tropicalis*) and lizard *Anolis carolinensis*. Searches to isolate the chicken TIP39 transcript and characterise its correspondent gene were also carried out following a similar strategy using the human (Q96A98) and zebrafish (AAI64665) sequences.

3.2.3. Sequence annotations and comparative analysis

The deduced mature peptide sequence of the chicken PTHRs (ckPTHRs) were obtained from BCM search launcher sequence utilities 6 frame translation option (http://searchlauncher.bcm.tmc.edu/seq-util). The receptor signal peptide sequence was predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP) and the localisation of transmembrane domain regions deduced using the TMHMM (http://www.cbs.dtu.dk/services/TMHMM) programme and were manually edited according to the PRINTS annotation (http://www.bioinf.manchester.ac.uk/dbbrowser/PRINTS/).

Multiple sequence alignments of the chicken PTHRs deduced amino acid sequences were performed using ClustalX [version 1.83 (Thompson, Gibson et al. 1997)] with the following parameters: Gonnet series matrix, Gap opening penalty 10, Gap extension 0.2. The alignments were displayed and manually edited and percentages of sequence similarity and identity calculated using GeneDoc (www.psc.edu/biomed/genedoc). Phylogenetic analysis was performed based upon the protein sequence alignments of selected PTHR and trees were constructed using the Neighbour Joining and Maximum Parsimony methods (Saitou and Nei 1987) with 1000

bootstrap replicates in MEGA 3.1 (Kumar, Tamura et al. 2004) and human secretin (HsaSCTR, AAA64949) as out-group.

3.2.4. Gene structure and gene linkage analysis

Characterisation of the *PTHRs* gene structures were performed on the basis of Ensembl gene predictions and by searching the chicken genome with the nucleotide sequences of the mature receptor precursor using the NCBI Spidey interface (http://www.ncbi.nlm.nih.gov/spidey/), and intron-exon boundary splice sites regions (AG/GT) identified were manually confirmed. The immediate gene environment of receptors were characterised using the NCBI genome chromosome annotation and compared with the homologue genome regions in human and zebrafish genomes that were accessed via the NCBI Mapview (http://www.ncbi.nlm.nih.gov/mapview/) and with the *Xenopus tropicalis* genome available from Ensembl (www.ensembl.org). Genes that were found to be predicted in the chicken *PTHRs* chromosomes that had no annotated homologue in the other 3 species were retrieved and used to query their genomes to confirm their absence.

3.2.5. Tissue distribution

RT-PCR was used to confirm the predicted PTHR identified *in silico* and to characterise receptor gene expression with specific primers (Table 4). Total RNA was extracted from 14 chicken adult tissues (forebrain, midbrain, hindbrain, duodenum, mid-gut, hind-gut, parathyroid, lung, kidney, liver, muscle, bone, cartilage, pituitary) and also from chicken embryos at different developmental stages (4HH; 6HH; 17HH; 26HH; 31HH; 36HH) and CAM from 44HH (Hamburger and Hamilton 1992) using the Tri Reagent (Sigma Aldrich, Spain) according to the manufacturer's instructions. Prior to cDNA synthesis, total RNA was denatured at 65ºC for 5 min, quenched on ice for 5 min and treated with DNAase using the DNA-free Kit (Ambion, UK). cDNA was synthesised using 2µg of DNase-treated tRNA with random oligonucleotides as follows: 20 µl reaction volume containing 10 ng of pd(N)6 random hexamers (GE Healthcare, UK), 2mM dNTPs (Promega, Spain), 100U of MMLV-RT (Promega, Spain) and 20U RNasin® Plus RNase inhibitor (Promega, Spain). Chicken cDNA was synthesized for 10 min at 20ºC followed by 50 min at 42ºC and 72ºC for 5 min.

PCR amplification reactions were carried out for a final volume of 25µl with 1.5mM $MgCl_2$ (Biocat, Germany), 0.2mM dNTP's (GE Healthcare, Spain), 0.25µM of receptor specific forward and reverse primers and 0.5U of EuroTaq DNA Polimerase (5U/µl, Euroclone, Italy) and cycled 30 times for *PTH1R* and 35 for *PTH3R*. To ensure the quality and quantity of the cDNA utilized in each reaction, a preliminary PCR for the amplification

of the 18S ribosomal unit with the primers *18Sfw* tcaagaacgaaagtcggagg and *18Srv* ggacatctaagggcatcaca was performed according to the cycle: 94ºC 3 min; (94ºC 30 sec; 55ºC 30 sec; 72ºC 45 sec) repeated 22 times, followed by 72ºC 10 min. The PCR products obtained were analysed on 1.5% agarose gels and the products of expected size were gel extracted and sequenced to confirm identity against the database.

To isolate a putative *PTHR2*, specific primers based upon conserved region of PTH2R from other vertebrates were designed (PTH2Rfw$_1$: *caaagtagttcatacacatataggagt*, PTH2Rfw$_2$: *tgcctcacacatttactgg*, PTH2Rrv: *ggactggctgctggtgct*) and PCR reactions were performed according to the condition described above using 35 cycles and annealing temperature of 55ºC.

Table 4: Primer pairs used to amplify the *ckPTH1R* and *ckPH3R* transcripts. The annealing temperatures of each primer-pair are given.

Receptor	***Tissue distribution***	***T (ºC)***	***Expression construct***	***T (ºC)***
PTH1R	*PTH1Rfw: atgggatcatatctggtttat* *PTH1Rrv: ggccagcagacaatacca*	*55*	*PTH1Rfw: atgggatcatatctggtttat* *PTH1Rfinalrv: ttacatcactgtctctctttc*	*57*
PTH3R	*PTH3Rfw: atggggtctgtgggcagg* *PTH3Rrv: gttgaagtcgtagatgtagtc*	*57*	*PTH3Rfw: atggggtctgtgggcagg* *PTH3Rfinalrv: tcatagcatcgtctccagct*	*59*

3.2.6. Expression vector constructs

The complete coding region including stop codon of *PTH1R* and *PTH3R* were amplified from embryo limbs (36HH) cDNAs with specific primers (Table 4). PCR was carried out using the Taq DNA proofreading polymerase (Advantage ® 2, polymerase mix, Clonetech) according to the manufacturer instructions with 10X Advantage 2 PCR Buffer, 0.2 mM dNTP's (GE Healthcare, Spain) and 0.25µM of each specific primers for a final volume of 25µl and amplified with the following parameters: 94ºC 2 min, (94ºC 30 sec, 57-59ºC 1 min, 72ºC for 2 min) x 30 cycles and 72ºC for 10 min. The PCR products obtained were gel extracted using the GFX -PCR DNA and Gel Band Purification kit (Amersham Biosciences, Spain) and the purified products were cloned into the pcDNA3.1/V5-His-TOPO expression vector (Invitrogen, USA) according to the manufacturer instructions. The recombinant clones obtained were PCR screened using vector and receptor specific primers combination and the bacterial colonies that contained the PTHRs in frame with the promoter vector pCMV were selected and plasmid DNA was extracted using the Midi-Prep kit (Roche). To facilitate receptor integration in the genome, approximately 5µg of the recombinant vector was linearized with the restriction enzymes *Apa*I and *Bgl*II (Promega)

and the digested product purified using the standard DNA purification phenol:clorophorm method and utilized to transfect human embryonic kidney cell line 293 (HEK293 from European Collection of Cell Cultures; ECACC, UK).

3.2.7. Cell transfection and receptor stable cell line production

HEK293 cells were maintained in complete Dulbecco's modified Eagle's medium (DMEM, Sigma, Spain) with 4500 mg/L glucose, 110 mg/L sodium pyruvate and L-glutamine and supplemented with 10% foetal bovine serum sterile filtered and 0.1% penicillin:streptomycin antibiotic mixture (10.000 U:10mg/ml , Sigma) in a humid 5% CO_2 incubator (Sanyo) at 37ºC. On the day prior to transfection, 2-3 x10^5 cells were plated in 6 well-plates (Sarsted, Portugal) and 0.2µg of the linearized constructs were added using the Fugene 6 (Roche, Germany) cell transfection reagent according to the manufacture protocol. The transfection complex was incubated 40 min at RT before being added to the cells which were left to grown with daily exchanges of complete medium. Selection of the stably transfected clones was performed 72 hours post-transfection with complete medium supplemented with 0.08µg/ml of Geneticin (G418 sulphate, Gibco) and 250 µg/ml sterile filtered 1:100 amphotericin B solution (Sigma, Spain). Cell death was found to be higher than 95% and cell recovery was monitored by constant changes of antibiotic selective medium and daily observation and the success of gene integration was confirmed by RT-PCR with receptor specific primers (Table 4).

3.2.8. cAMP assay

Peptide potency profile of the chicken PTH-family members was tested on the PTHRs stable cell lines by measuring the production of intracellular cAMP. Three independent experiments were carried out and the PTHRs were assayed at the same time. Two days prior the assay, 2-3x10^5 cells were plated on 96 well/plates and stimulated in the presence of a decreasing concentrations (100nM to 0.1nM) of PTH(1-34), PTHrP(1-34) and PTH-L(1-34) and also with 100nM of human PTH(1-34) and TIP39 (Sigma, Spain).

Prior to the assay, cells were incubated at 37°C for 40 min in a CO_2 incubator with 1mM of IBMX (3-Isobutyl-1-Methylxantine, Sigma, a phosphodiesterase inhibitor) followed by the addition of fresh medium containing the peptides in the presence of 1mM IBMX for an extra 40 min period. Forskolin (an inductor of cAMP production; 0.1mM) was used as positive control and assay negative controls were carried out using non-transfected cells in the presence or absence of peptide highest concentration (100nM). Cells were washed and resuspended in 100µl of 1xPBS/0.5M EDTA and immediately frozen at -80°C to promote cell lysis for later quantification of intracellular cAMP production.

3.2.9. Radioimmunoassay (RIA)

Cells were lysed using 3 consecutive thaw (42ºC)/freeze (-80ºC) cycles and the supernatant and cell debris were transferred to eppendorf tubes and sonicated for 20 sec on ice. Cells were boiled at 100ºC for 10 min to denature proteins and the supernatant was collected after centrifugation for 10 min at 4ºC and 19.000 G. cAMP was quantified by radioimmunoassay using the TRK432 kit (GE Healthcare, UK) following the manufacturer's instructions. Concentration of cAMP (pmol/well) was determined for each sample in duplicate and calculations were performed based on a linear regression curve constructed with standard concentrations of cAMP. Basal cAMP accumulation from each peptide negative controls was subtracted from the corresponding treatments and production above basal levels per well (cAMP/well) was plotted against peptide concentration. cAMP was also quantified in HEK293 cells stably transfected with the pCMV-GFP (vector expressing the Green fluorescent protein) and results were equivalent to the peptide assay negative controls and the presence of a vector construct in the cells did not show to interfere with cell cAMP production.

3.2.10. Measurement of intracellular Ca^{2+} influx

Intracellular Ca^{2+} was measured using the Ca^{2+} sensitive fluorescent dye Fluo-4 NW according to manufacturer's instructions (Molecular Probes, Invitrogen). Prior to the assay, plates were treated with sterile Poly-L-lysine (0.1mg/ml) to avoid cell release. Approximately $5x10^4$ cells in 100 µl of selective medium were plated per well in 96 well flat bottom plates (half area with black walls, Greiner, Germany) and allowed to attach for 2 days. Medium was removed and cells were washed once with 2.5mM probenecid (Molecular Probes) in 1xPBS and incubated for 30 min at 37ºC with 100µl of the Fluo-4 NW dye followed by additional 30 min incubation period at room temperature. The dye was removed and the different peptides in decreasing concentrations (from 0.1nM to 100nM) were added and fluorescence was measured immediately every 10 seconds for 2 minutes using a Synergy4 (Biotek, USA) plate reader. Carbachol (100nM; Sigma, Spain) was used as the positive control of the assay and background signal was determined by measuring fluorescent in plate wells using non-transfected cells. For assay negative control, non-transfected cells were incubated with the peptide highest concentrations.

3.2.11. Statistics

The results are presented as the mean ± SEM of three independent experiments carried out in duplicate. Data was ploted as the output in cAMP or Ca^{2+} at different peptide concentrations for each PTHR using SigmaPlot 9 (Systat, Inc., San Jose, CA). The EC50

and confidence limits were calculated using a sigmoidal curve fitting within the Single ligand binging routine of the pharmacology model of Sigmaplot. Unfortunately, the only radioimmunoassay for cAMP was withdrawn from the market while the study was already advanced and it was not possible to extend the concentration range tested, and saturation was not achieved with some of the peptides. To improve curve fitting we considered the maximum empirical stimulation achieved for each receptor as the maximum at e^{-5} molar for all assays and fitted the curve that way.

3.3. Results

3.3.1. The chicken PTH/PTHrP Receptors

In the chicken genome two potential *PTHR (*ENSGALT00000008796/XM_418507 and ENSGALT00000031444/XM_425837) were identified and comparative analysis of their deduced mature protein coding sequence revealed they share 76% and 90% sequence similarity with other vertebrates PTH1R and 72% with the zebrafish PTH3R, respectively (Figure 22). The deduced sequence of the putative *PTH1R* mRNA was confirmed by RT-PCR on chicken cDNA from whole embryos at stage 26HH using specific primers and by the identification of 3 incomplete ESTs (BU219643 obtained from stage 20-21HH whole chick embryos, BU401969 isolated from stage 36HH limbs, and BU419888 from growth plates chondrocytes). The deduced sequence of the *PTH3R* was also confirmed by RT-PCR using cDNA from whole chicken embryo at stage 26HH and searches of the chicken EST database failed to identify putative transcripts. Despite *in silico* database searches coupled to DNA amplification techniques it was not possible to identify and isolate a potential *PTH2R* receptor gene or transcript.

The chicken *PTH1R* and *PTHR3* receptors coding sequences are 1614bp and 1626 bp in length, corresponding respectively to deduced proteins of 538 and 542 amino acids (Figure 22) and they share 76% overall sequence identity. Moreover, searches performed on the chicken genome and EST databases failed to identify the presence of a homologue of the vertebrate TIP39.

3.3.2. Sequence comparisons

In the genomes of *Xenopus tropicalis* and lizard partial sequences of putative *PTH1R*, *PTH2R* and *PTH3R* receptors were identified (Table 5 and Table 6).

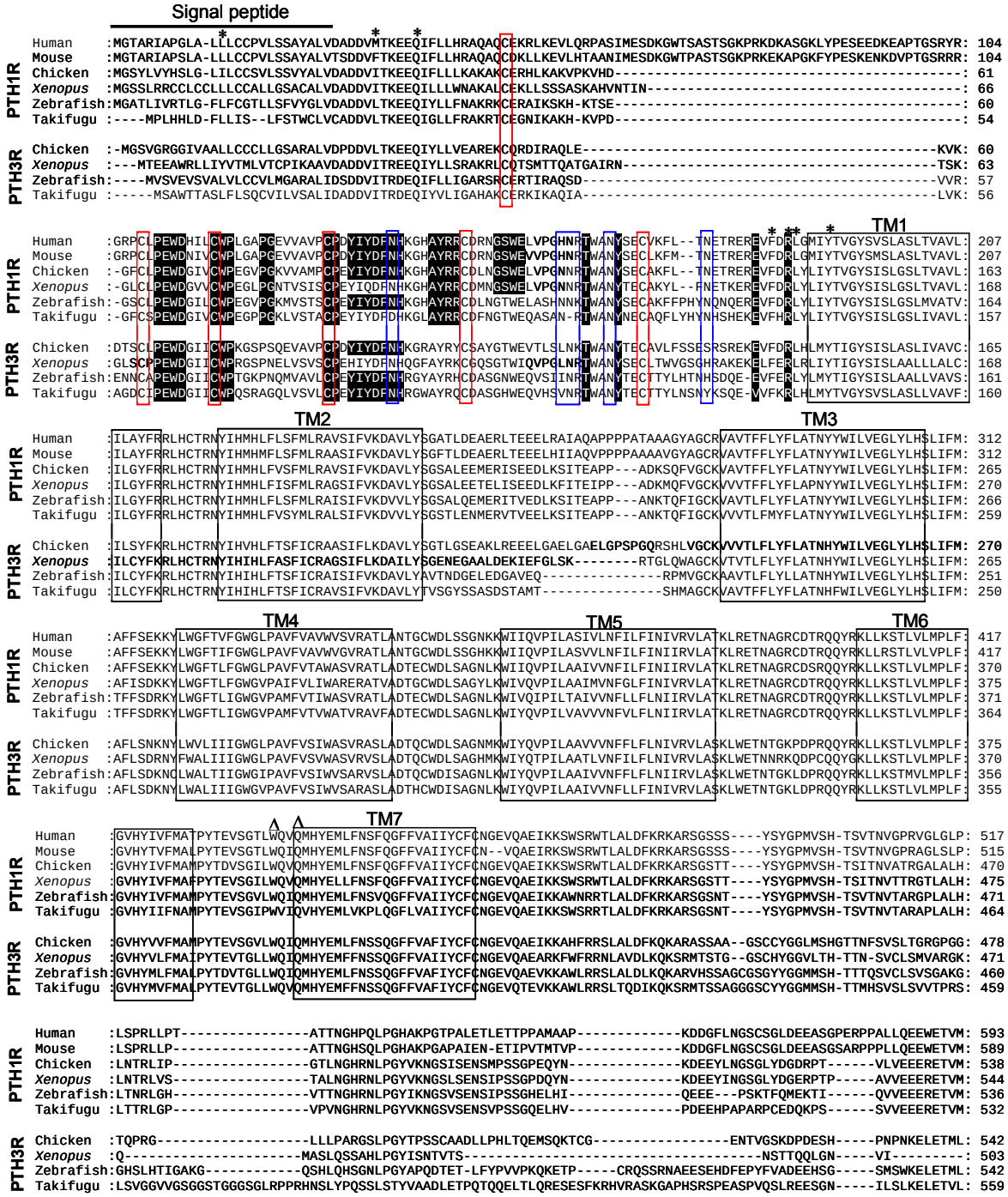


Figure 22: Multiple sequence alignment of the chicken and other vertebrates PTH1R and PTH3R. The seven transmembrane domains (TM) are annotated and represented by boxes and the signal peptide sequence by a line. "*" and "Δ" indicate amino acid residues involved in ligand binding at the N-terminal and within TM regions, respectively ((Gardella and Juppner 2001) (Gardella, Juppner et al. 1994). The cysteine residues and putative N-glycosylation sites are within red and blue boxes, respectively. Amino acid motifs that were found to be determinant for PTH binding are annotated in black (Cardoso, Pinto et al. 2006). Accession numbers are in Table 5.

Moreover searches performed in other avian genomes of the zebra finch (*Taeniopygia guttata*) and turkey (*Meleagris gallopavo*) also identified homologues of the chicken *PTH1R* and *PTH3R* genes and failed to retrieve *PTH2R*. In the invertebrate genomes of *Ciona* (XM_002121230) and amphioxus (XM_002599399) putative PTHR receptor sequences with at least 52% and 47% similarity to the chicken homologues were also identified.

Multiple sequence alignments of the chicken PTHRs deduced mature peptide sequences with their vertebrate homologues revealed that the deduced receptor proteins are composed of seven transmembrane domains (TM) and six conserved cysteine residues at the N-terminal region (Figure 22). Conserved positions for putative N-glycosylation sites were also present and 4 were identified in chicken PTH1R and 3 in chicken PTH3R. Within this region, the residues L^{13}, T^{33}, Q^{37}, F^{184}, R^{186}, L^{187} and I^{190} which have been previously identified to be involved in the interaction of the mammalian PTH1R with PTH(1–34) and PTHrP(1–34) (Juppner, Schipani et al. 1994; Gardella and Juppner 2001) are also present in the chicken PTHRs with the exception of L^{13} which is replaced by I^{13} in PTH1R and I^{190} which in PTH3R was substituted by M^{190} (Figure 22).

Table 5: Protein accession numbers (GenBank and Ensembl) of vertebrate PTHRs. "*" indicates the sequences identified in this study.

	PTH1R	**PTH2R**	**PTH3R**
Human	AAR18076	AAH36811	Not identified
Mouse	NP_035329	NP_644676	Not identified
Chicken	FR746109*	Not identified	FR746110*
Zebra finch	XP_002191985*	Not identified	XP_002191438*
Turkey	ENSMGAP00000002429*	Not identified	ENSMGAP00000001447*
Lizard	ENSACAP00000004707*	ENSACAP00000010946*	XM_003222677*
Xenopus	ENSXETP00000007949	ENSXETP00000017570	ENSXETP00000007049
Zebrafish	NP_571432	NP_571452	NP_571453
Takifugu	CAD79707	CAD68048	CAD67555

Note: "*" indicates the sequences identified in this study.

In addition, the motifs residues D^{113}, W^{118}, P^{132} and W^{154} which are involved in the conformation of the ligand-binding pocket of family 2-GPCR B1 receptors (Cardoso, Pinto et al. 2006) are also present in chicken sequences (Figure 22).

Table 6: Percentages of amino acid sequence similarity/identity of the non-mammalian tetrapod and invertebrate PTHRs with the chicken and human homologues.

		Human		Chicken	
Organism	Receptor	PTH1R	PTH2R	PTH1R	PTH3R
Xenopus	PTH1R	*63/73*	*46/62*	***83/89***	*52/65*
	PTH2R	*42/57*	***70/81***	*47/64*	*44/61*
	PTH3R	*45/59*	*42/57*	*50/64*	***56/67***
Lizard	PTH1R	*66/76*	*44/58*	***74/82***	*49/61*
	PTH2R	*42/56*	***70/80***	*47/63*	*43/61*
	PTH3R	*46/58*	*41/59*	*52/66*	***60/70***
Zebra finch	PTH1R	*66/76*	*43/58*	***84/85***	*49/61*
	PTH3R	*47/61*	*42/60*	*53/68*	***77/83***
Turkey	PTH1R	*66/75*	*48/65*	***95/95***	*53/67*
	PTH3R	*47/56*	*41/57*	*52/62*	***83/84***
Ciona	PTHR	*36/49*	*37/63*	*40/56*	*37/52*
Amphioxus	PTHR	*31/44*	*32/46*	*34/49*	*33/47*

Note: Accession numbers are indicated in Table 5 and in bold the highest percentages obtained are highlighted.

3.3.3. Phylogenetic analysis

Phylogenetic analysis of the vertebrate PTHRs is shown in Figure 23.

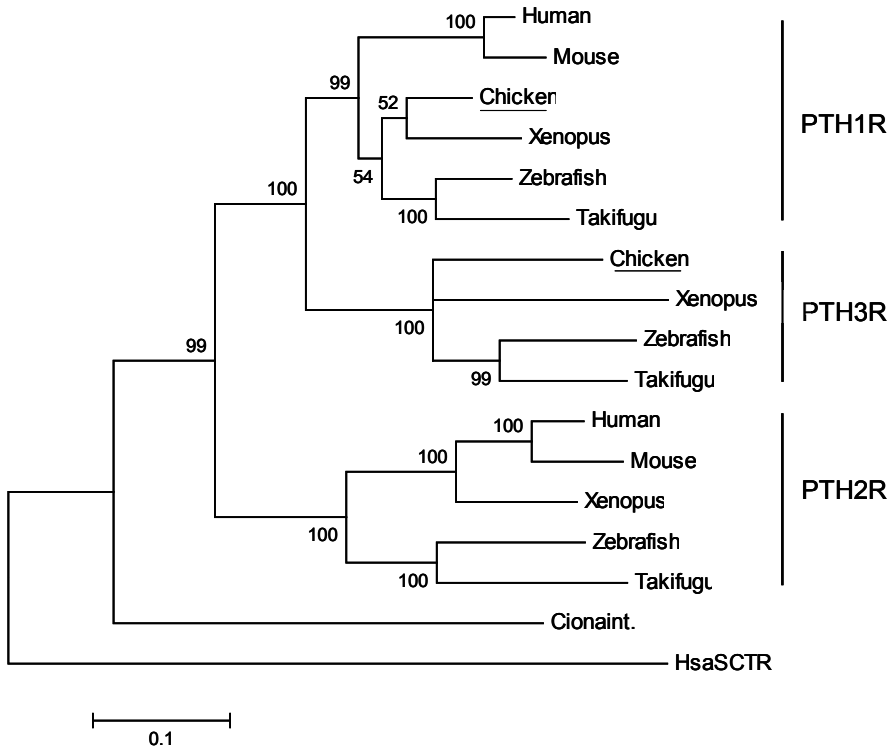


Figure 23: Consensus phylogenetic tree of the PTHRs constructed using the Neighbor Joining method (Saitou and Nei 1987) and 1000 bootstraps replicates in Mega3.1 software (Kumar,

Tamura et al. 2004). The tree was constructed according to the parameters: pairwise deletion, p-distance model and 222 number of informative sites. The ckPTH1R and ckPTH3R are underlined and the sequence of the human secretin receptor (HsaSCTR, AAA64949) was used as out-group. Accession numbers in Table 5.

The consensus tree obtained suggests that members of this family share a common ancestor at the deuterostome radiation and have evolved via gene or genome duplication events in the vertebrate lineage. Two major clades are presented, one containing the vertebrate PTH2R and the PTH1R and PTH3R clusters. This suggests that after initial duplication they have undergone distinct evolutionary pressures and that PTH1R and PTH3R are the result of a more recent duplication event. The chicken PTHRs identified, group within the PTH1R/PTH3R branch (Figure 23) confirming their identity. Despite extensive searches a putative PTH3R gene homologue was not identified in mammalian taxa.

3.3.4. Gene structure and short-range gene linkage analysis

The chicken *PTH1R* and *PTH3R* share an identical gene structure composed by 13 exons and identical to the predicted gene structures of their homologues in *Xenopus* and zebrafish. This contrasts to human in which *PTH1R* gene organisation comprises 15 exons within the mature receptor region (data not shown) (Rubin, Hellman et al. 1999). For both chicken *PTH1R* and *PTH3R*, the signal peptide region is encoded in the 1st exon and the TM regions are distributed between exon 5th to exon 13th (Figure 22, Additional Figure 1, Additional Figure 2).

The gene environment of the chicken *PTHRs* was compared with the homologue genome regions in zebrafish, *Xenopus* and human. Gene synteny was maintained for both receptors. The *PTH1R* gene maps to chicken chromosome 2, to human chromosome 3, to *Xenopus* scaffold 479, and to zebrafish chromosome 2 and one linked gene was identified: *TMIE* (transmembrane inner ear-like) (Figure 24). However, the analysed region in zebrafish is 5 times longer than the corresponding region in tetrapods. The chicken *PTH3R* gene maps to chromosome 27 and its homologue to *Xenopus* scaffold_155 and zebrafish chromosome 12. The gene *CCDC44* (coiled-coil domain-containing protein 44) was identified in the genome regions analysed although is localised in closer proximity within the tetrapod gene environment.

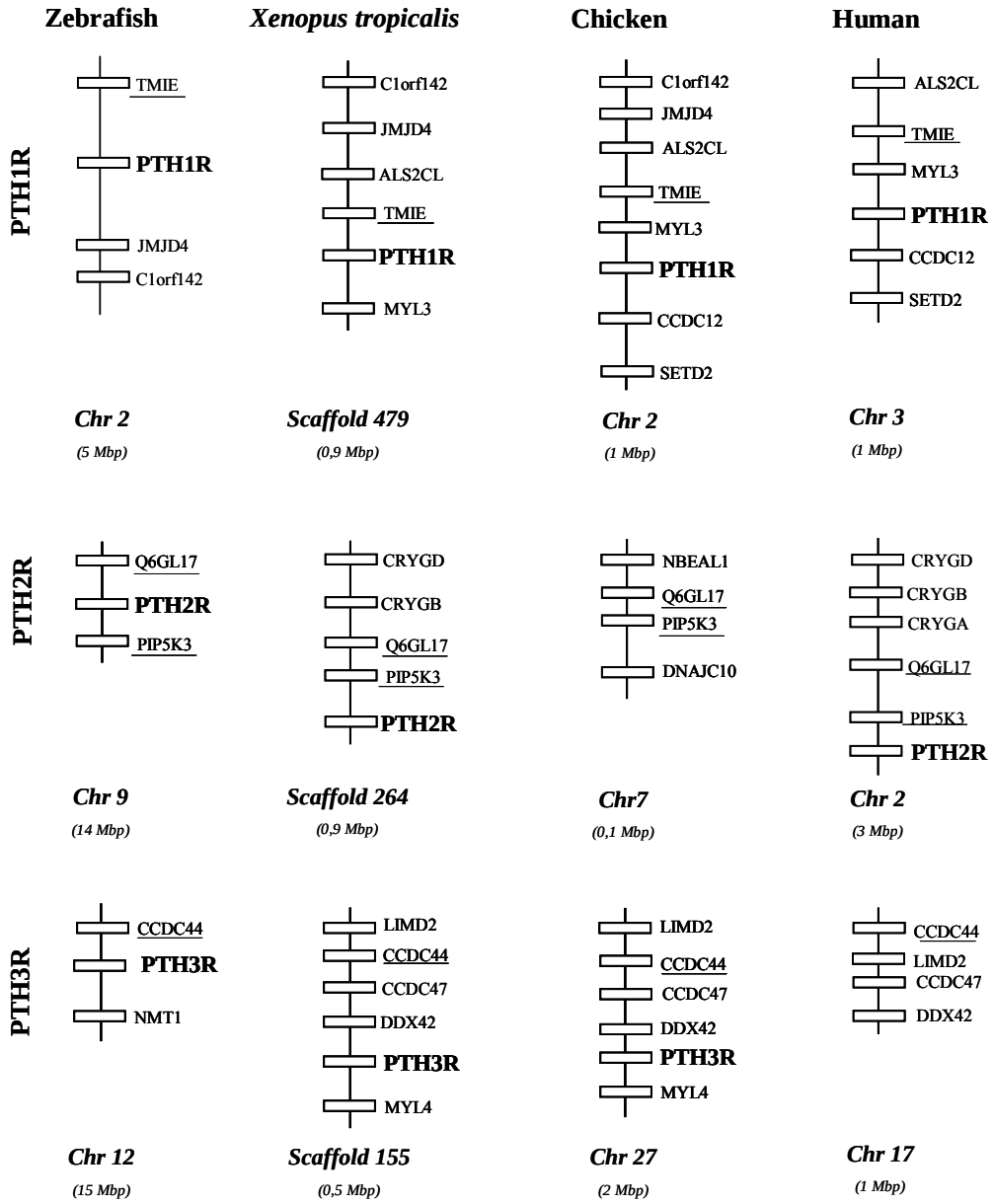


Figure 24: Short-range gene linkage analysis of the chicken *PTHRs* genome environment with zebrafish, *Xenopus* and human homologue regions. Genes are represented by closed boxes and the size of the chromosome region analyzed is given within brackets. Genes were named according to the HUGO annotation and lines indicate chromosome/scaffolds segments. The *PTHR* genes are highlighted in bold and conserved linked genes are underlined. For simplicity, only synteny genes are represented and the figure is not drawn to scale.

In the human genome, homologues of the conserved vertebrate *PTH3R* gene environment were identified in chromosome 17 despite the absence of receptor locus. Similarly, the genes *Q6GL17* and *PIP5K3 (*1-phosphatidylinositol-4-phosphate 5-kinase) found in close proximity to the vertebrate *PTH2R* were also identified in the chicken chromosome 7 despite the absence of *PTH2R*. The genome environment of the

vertebrate *TIP39* gene was also characterised and no gene homologues were identified in the chicken genome (data not shown).

3.3.5. Tissue distribution

The distribution of the *chicken PTHRs* transcripts was characterised on chicken adult tissues and embryos at different stages of development (Figure 25).

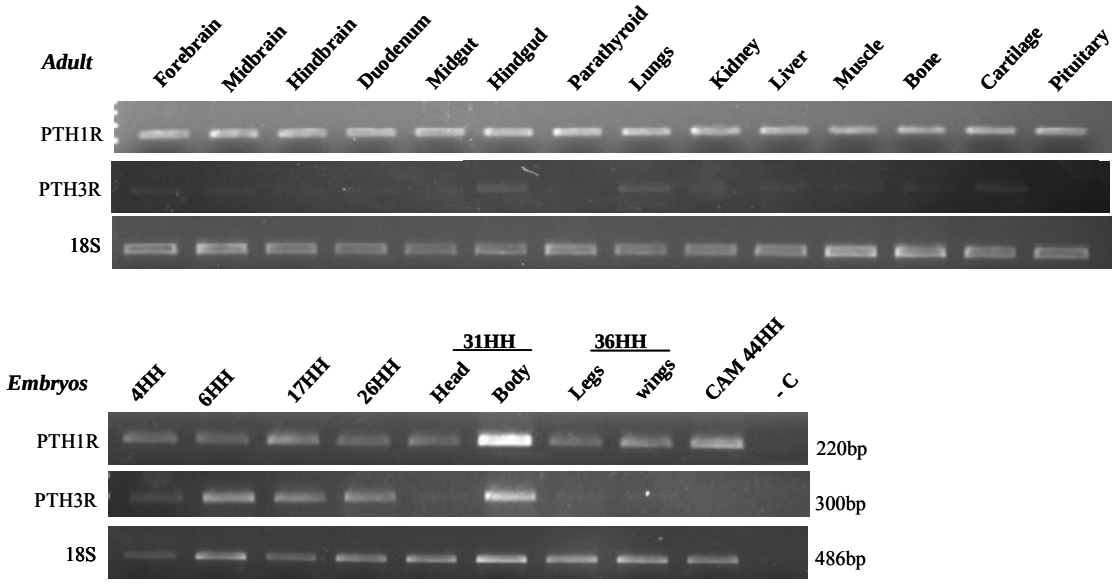


Figure 25: Expression profile of the chicken *PTH1R* and *PTH3R* by RT-PCR in adult tissues and different embryonic stages which are represented by the different Hamburger and Hamilton stages (Hamburger and Hamilton 1992). The 18S amplification control is shown for each tissue and the amplicon size obtained is given in base pairs (bp).

In general, both receptors were found to be present in the majority of the tissues sampled in adult and chick embryonic tissues and are expressed from early stages of development. During chicken embryonic development, both receptors start to be expressed from 19 hours of incubation (4HH) and are continuously expressed in the subsequent stages analysed however, *PTH3R* was absent or down-regulated in the head of 31HH and 36HH. In the CAM of stage 44HH the *PTH1R* was the only receptor amplified (Figure 25).

3.3.6. cAMP production

The cAMP accumulation of transfected cells expressing *chicken PTH1R* and *PTH3R* in the presence of different concentrations of PTH(1-34), PTHrP(1-34) and PTH-L(1-34) is shown in Figure 26. All chicken peptides were able to activate the two receptors in a dose-dependent manner with different half maximal cAMP response (EC50) values.

PTHrP stimulation of PTH1R at 10^{-7} M (17.21±1.84 pmol/well, $p<0.05$) was significantly greater (twice) than PTH-L (9.13±1.44 pmol/well) and PTH (6.03±0.78 pmol/well).

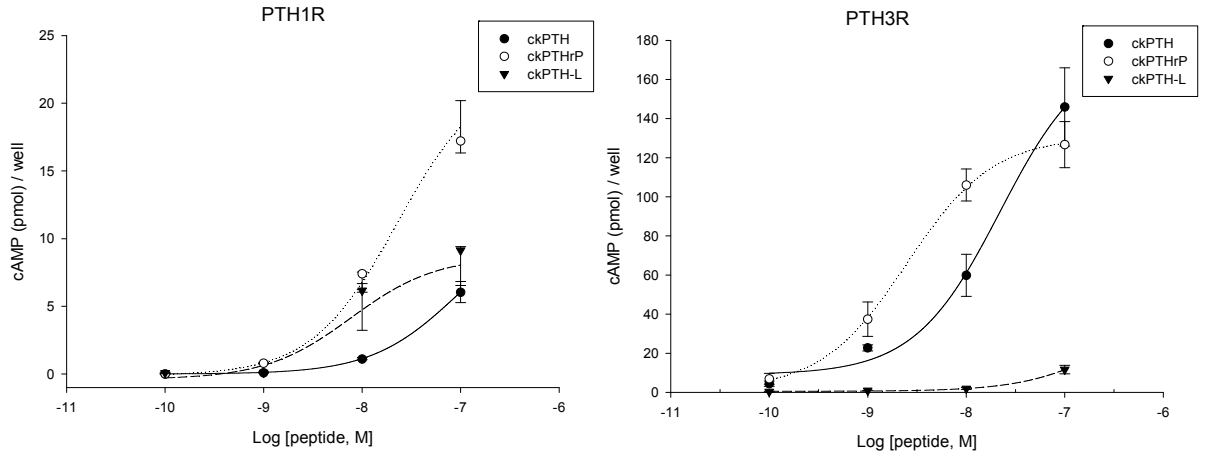


Figure 26: Accumulation of cAMP in HEK293 cells transfected with ckPTH1R and PTH3R stimulated with decreasing concentrations of the (1-34aa) ckPTH, ckPTHrP and ckPTH-L. Values represent means ± SEM of three independent experiments.

Stimulation of PTH3R at 10^{-7}M was highest for PTHrP (119.93±11.76 pmol/well) and PTH (144.72±20.02 pmol/well), while PTH-L produced a small cAMP stimulation above basal concentration (12.59±2.13pmol/well, $p<0.05$),approximately 13 times lower than PTH and PTHrP. The peptide potency profile based upon EC50 values reveal no statistically significant difference (Table 7).

Table 7: cAMP production by chicken PTH1R and PTH3R in presence of the chicken 1-34 PTH-family peptides.

	PTH1R			***PTH3R***		
	EC_{50} (nM)	*95% Confidence Intervals (nM)*	*E_{10}^{-7} (pmol/well)*	*EC_{50} (nM)*	*95% Confidence Intervals (nM)*	*E_{10}^{-7} (pmol/well)*
PTHrP	14.4*	8.2 - 25.4	17.21±1.84 *	6.80	0.03 - 15.4	119.93±11.76
PTH	202	163 - 249	6.03±0.78	26.5	14.0 - 50.0	144.72±20.02
PTH-L	120	54.1 - 268	9.13±1.44	1913*	1469 - 2491	12.59±2.13 *

Note: Maximum cAMP stimulation at 10^{-7} M is indicated by E_{10}^{-7} (mean ± SEM) and the "*" indicates statistical significance ($p < 0.05$).

The human PTH was also able to stimulate the chicken PTH1R at an apparent level similar to chicken PTHrP (Figure 27). The human TIP39 demonstrated negligible accumulation of cAMP production in both PTH1R and PTH3R (Figure 27).

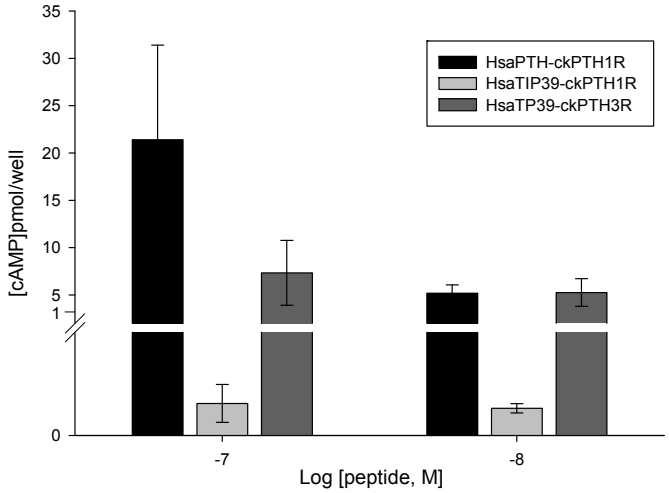


Figure 27: Accumulation of cAMP in HEK293 cells transfected with chicken PTH1R and PTH3R stimulated with 100nM and 10nM of the Human PTH and TIP39 peptides (HsaPTH, HsaTIP39). Values represent means ± SEM of a single experiment carried out in duplicate.

3.3.7. Ca^{2+} accumulation

A preliminary peptide screening using 1μM and 100nM of each peptide revealed that PTH3R did not activate this signalling pathway and no changes in intracellular Ca^{2+} (iCa^{2+}) were observed. The capacity of PTH1R to stimulate iCa^{2+} was further explored with decreasing concentrations (1μM to 0.01nM) of the chicken PTH-family members. Only PTHrP was able to stimulate the iCa^{2+} and PTH and PTH-L cause a negligible stimulation (Figure 28).

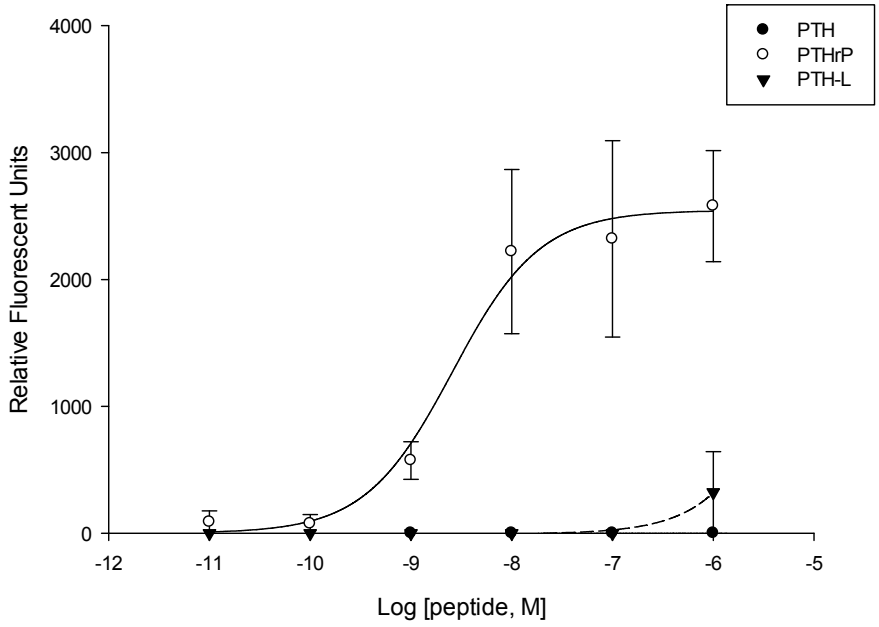


Figure 28: Accumulation of iCa^{2+} in HEK293 cells transfected with PTH1R and stimulated with decreasing concentrations of the chicken (1-34aa) PTH family peptides (PTH, PTHrP, PTH-L). Values represent means ± SEM from three independent experiments performed in triplicate

3.4. Discussion

In this study, two chicken PTHRs, homologues of the vertebrate *PTH1R* and teleost fish *PTH3R* genes, were isolated and shown to be widely expressed and activated by PTH family peptides.

The chicken PTH1R and PTH3R amino acid sequence share at least 72% of similarity with vertebrate homologues and contain conserved amino acid motifs previously identified to be implicated in receptor ligand-binding. The six cysteine residues present in the mammalian PTH1R, responsible for the three proposed disulfide bonds which occur between positions C^{48}/C^{117}, C^{108}/C^{148}, and C^{131}/C^{170}, originating a complex tertiary structure in the N-terminal domain (Gensure, Gardella et al. 2005) are also present in both PTHRs (Figure 22). Moreover, the presence of other amino acid residues involved with ligand-binding interaction at the N-terminal domain and also the Trp^{437} and Gln^{440} (Gardella, Juppner et al. 1994) located in the third extracellular suggests a potential conserved functional structure of the chicken PTHRs with the human PTH1R (Lee, Luck et al. 1995; Gardella and Juppner 2001). Chicken receptor gene structure and gene environment are also conserved across vertebrates, with the exception of the human *PTH1R* structure. Chicken *PTH1R* genomic structure is similar to the fish and amphibian while the human has an exon 2, which correspond to a part of an extracellular loop, and the presence of an extra exon (exon 15) at the C-terminal region, as a result of an intron gaining.

Members of the PTHRs family emerged early in the deuterostome radiation as revealed by the identification of sequence homologues in *Ciona* and amphioxus genomes (Kamesh, Aradhyam et al. 2008). The presence of chicken *PTH1R* and *PTH3R* genes are the result of a duplication process which occurred early in the vertebrate lineage prior to teleost divergence. Previously *PTH3R* had only been identified in teleost fishes and was suggested to be a specific duplication of *PTH1R* (Rubin, Hellman et al. 1999; Rubin and Juppner 1999). However, the isolation of the chicken homologue and the identification of *PTH3R* in Amphibia suggest a different model of receptor evolution in which three PTHRs originated early in vertebrate evolution, prior to the vertebrate branch that gave rise to the tetrapods and teleost fishes. Despite the identification in chicken of genes associated with the vertebrate *PTH2R* gene environment, the receptor was not identified in chicken and in other birds, which suggests that *PTH2R* was eliminated from the avian lineage. Moreover, the *TIP39* gene, which product is a potent stimulator of the mammalian and teleost PTH2R (Hoare, Rubin et al. 2000) seems to have followed an evolutionary trajectory similar to *PTH2R*. At present the function of the vertebrate TIP39/PTH2R system is still

poorly understood and seem to be involved in several physiological processes especially in the nervous system (Della Penna, Kinose et al. 2003; Dobolyi, Palkovits et al. 2010), including the modulation of SOX9 expression and inhibition of chondrocyte proliferation and differentiation (Panda, Goltzman et al. 2009), and the participation in suckling-induced prolactin release (Cservenak, Bodnar et al. 2010).

The chicken *PTH1R* and *PTH3R* are expressed in a large range of tissues including the classical calcium-target tissues. *PTHR1* was found to be expressed at high levels in kidney of humans (Schneider, Feyen et al. 1993), however also with a widespread expression in a range of tissues (Usdin, Gruber et al. 1995). In fish was reported to be mainly expressed in the liver, gonad, skin, brain and pituitary (Hang, Power et al. 2005; Guerreiro, Renfro et al. 2007) and in *Xenopus* in lungs, brain, skin, kidney and bone (Bergwitz, Klein et al. 1998). Although *PTH3R* is at present poorly characterised, in sea bream it is expressed in the intestine (Rotllant, Guerreiro et al. 2006). In chicken it was found to be mostly expressed in the hindgut and kidney, suggesting a potential role in calcium homeostasis. Moreover, the chicken receptors were expressed during early embryo development when bone/cartilage structures develop which is consistent with previous suggestions of a putative role of PTHrP and PTH1R in chicken mandibular process (Zhao, Brauer et al. 2002). The calcium for this process is likely to originate from the egg CAM (Pinheiro, Cardoso et al. 2010) and is likely mediated via *PTH1R*, since it was the only receptor amplified from this tissue.

The peptide potency profile of the chicken receptors revealed that they are activated by the ckPTH-family members in a dose-depend manner. Of the two receptors PTH3R accumulated one order of magnitude higher cAMP than PTH1R, while only PTH1R seemed to signal through Ca^{2+}. Studies with the zebrafish PTH1R in which human PTH and PTHrP were able to activate IP3 pathway also support this observation (Rubin and Juppner 1999). The PLC/iCa^{2+} pathway in mammals is involved in several processes including bone formation and muscle contraction (Fukami 2002). In chick, tibial growth plate chondrocytes require protein kinase A stimulation (Zuscik, O'Keefe et al. 2002) and a role for PTH1R seems to be confirmed by the expression of this receptor, and not PTH3R, in developing legs. Considering that there is a large tissue overlap of gene expression between the two receptors and that PTHrP seems to be the preferred ligand to activate both receptors, this observation highlights the possibility of different mechanisms of action and functional responses to the same ligand, and a complexity of ligand-receptor interactions and multiple receptor responses in the same cell. Furthermore, in chicken 5 distinct *PTHrP* transcript isoforms with identical 1-34 N terminal domains were recently identified which appear to have specific distributions in tissues and therefore potentially different physiological roles (Pinheiro, Cardoso et al. 2010). Furthermore, several other

signalling pathways, such as mitogen-activated protein kinase, also appear to be involved [reviewed by (Gensure, Gardella et al. 2005)].

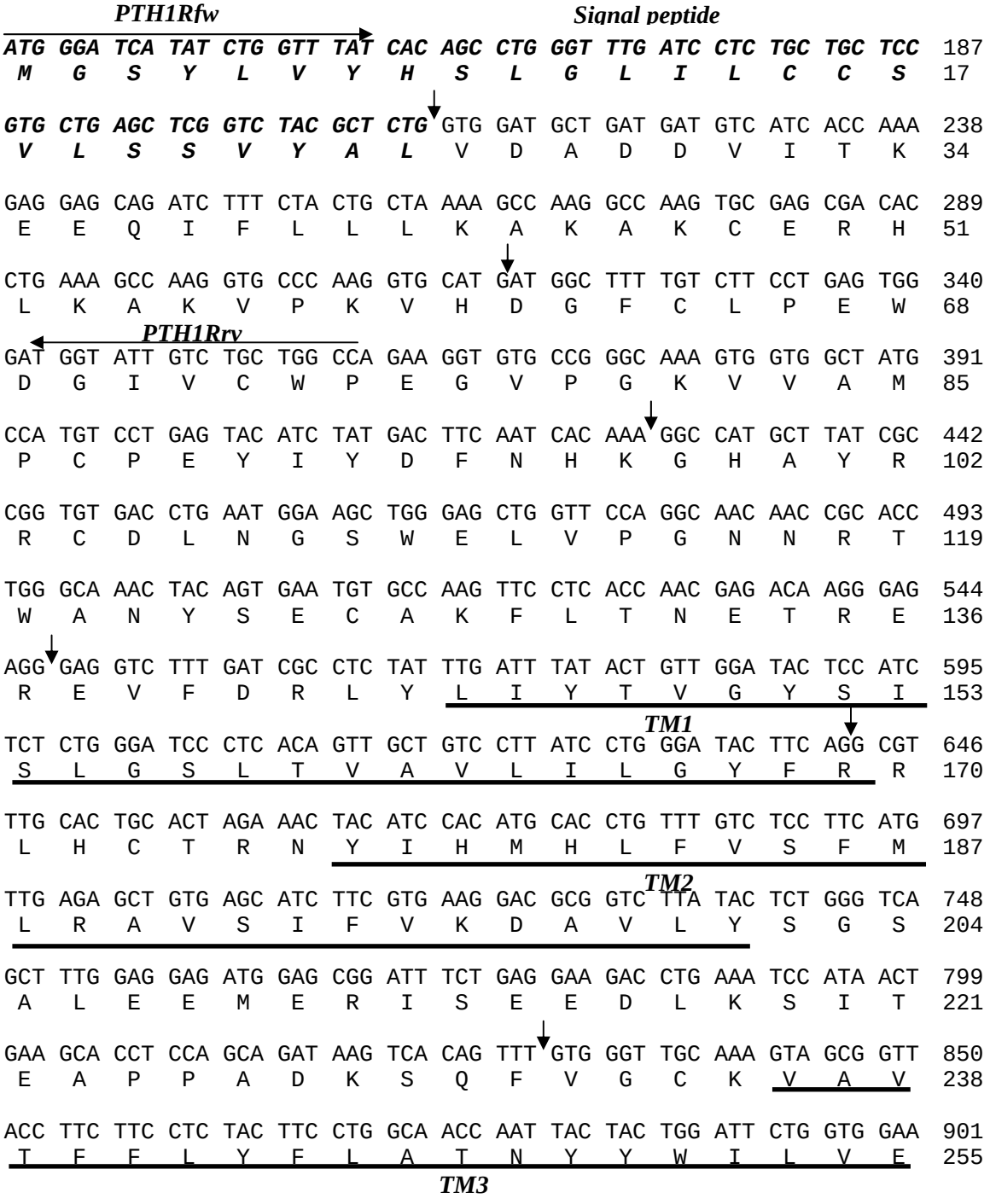
PTHrP was the most potent to stimulate cAMP accumulation in both PTH1R and PTH3R and only overlapped in potency with PTH in the latter receptor. This contrasts with zebrafish receptors, in which Fugu PTHrPA, human PTHrP and PTH were indistinguishable in their potencies to activate zfPTH1R while the zPTH3R was most efficiently activated by hPTHrP and FuguPTHrP and hPTH was approximately 22-fold less potent (Rubin and Juppner 1999). Although the potency profile of the human peptides was not completely characterised, preliminary data suggest that for PTH1R the human PTH stimulates cAMP production in an apparent similar potency to chicken PTHrP. Interestingly, PTH-L has low capacity to activate any of the receptors which appears to be consistent with a low calciotropic activity in chicken (Pinheiro, Cardoso et al. 2010). Whether other receptors exist for the parathyroid family of peptides as suggested by others [e.g., (Gensure, Gardella et al. 2005)] still needs to be found.

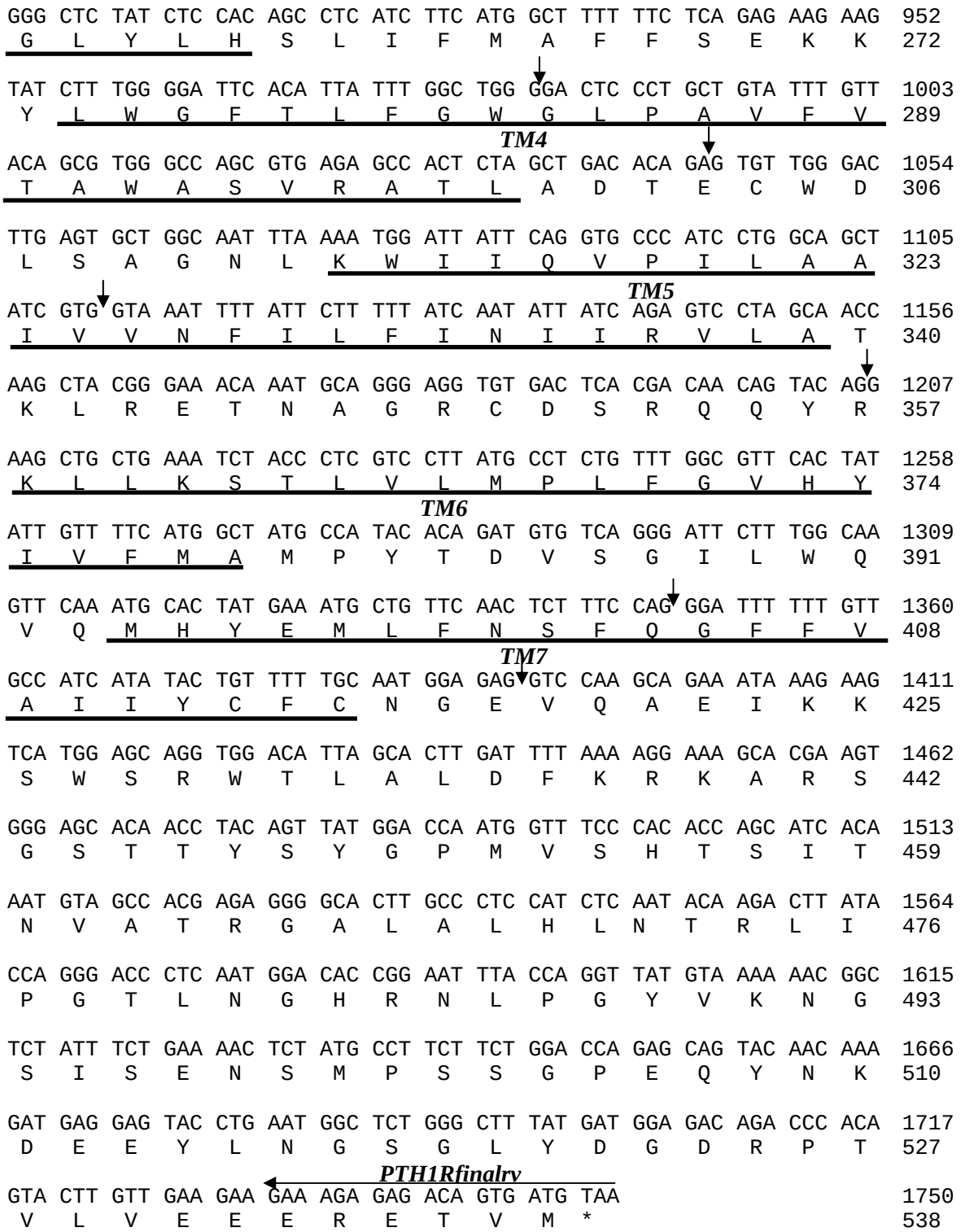
Interestingly, despite its absence from the chicken genome, human TIP39 was able to stimulate cAMP which is probably related the similarity of the secondary structure to PTH/PTHrP (Hoare and Usdin 2001) and receptor conservation.

In conclusion, two *PTHRs* were isolated in chicken, one of which was initially thought to be absent from tetrapods. In contrast, *PTH2R* and *TIP39* appear to be absent from the bird lineage. The pattern of expression of the receptor genes during development and in different tissues, and their activation response to the potential ligands suggest a complex mechanism and multiple functions for which more than one receptor may contribute.

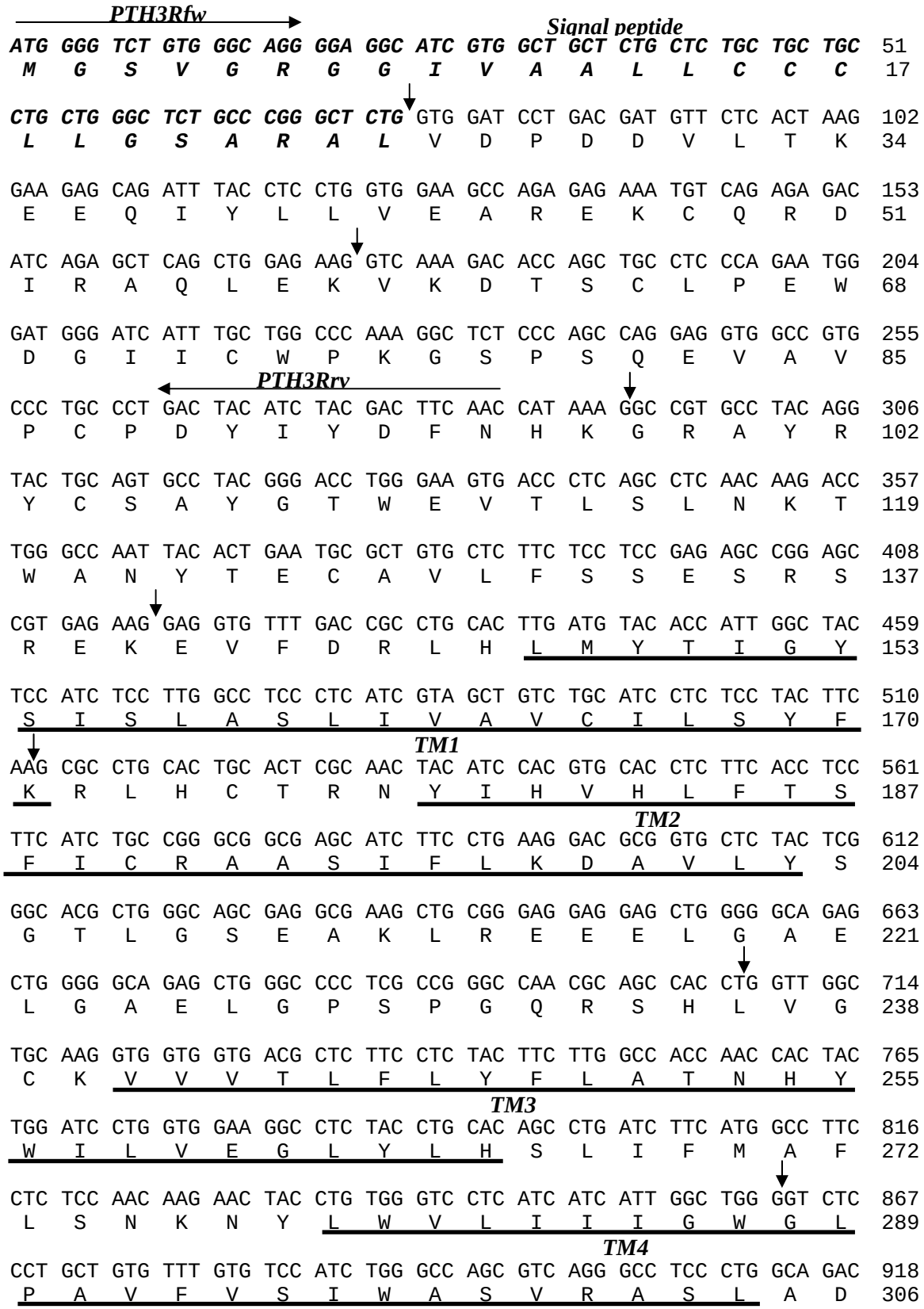
3.5. Additional figures

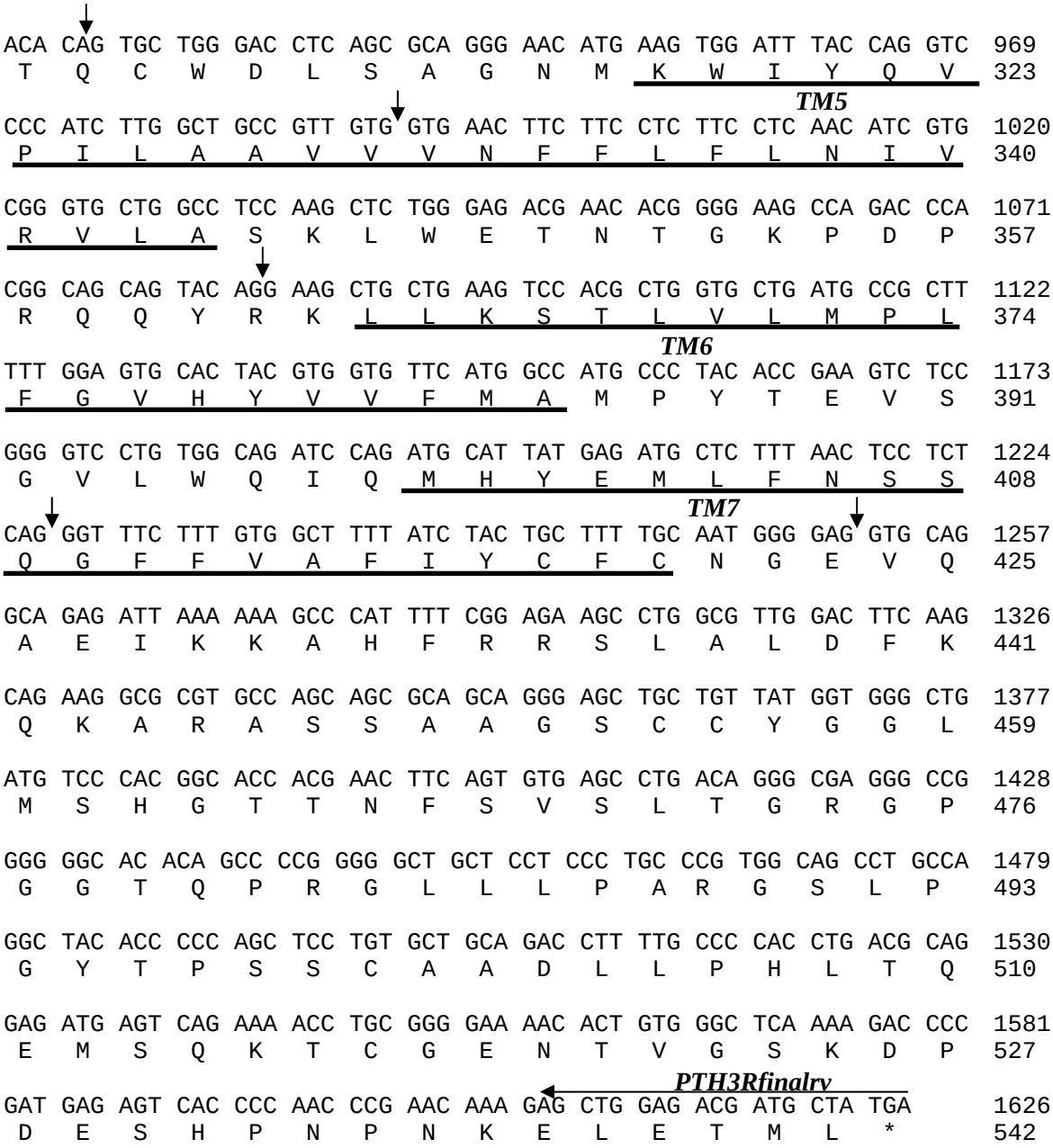
Additional Figure 1: Nucleotide and deduced amino acid sequence of the chicken PTH1R. Sequence deduced based upon EST data and PCR amplification. The primers localization are represented by horizontal arrows and the exons change by vertical arrows. The TM domains are represented by bound lines and signal peptide in italic and bold.




TM4
TM5
TM6
TM7
PTH1Rfinalrv

Additional Figure 2: Nucleotide and deduced amino acid sequence of the chicken PTH3R. Sequence deduced based upon EST data and PCR amplification. The primers localization are represented by horizontal arrows and the exons change by vertical arrows. The TM domains are represented by bound lines and signal peptide by in italic and bold.




TM5
TM6
TM7
PTH3Rfinalrv

CHAPTER IV

Ontogenic Gene Expression of PTH-family Members in Chicken

With: Helen Downie, João CR Cardoso, Cheryll Tickle, Deborah M. Power and Adelino V.M. Canário

Abstract

Parathyroid hormone (PTH) from the parathyroid glands regulates calcium homeostasis. A related peptide, PTHrP, is a paracrine/autocrine factor which regulates calcium, bone differentiation and morphogenesis and activates with PTH a common G protein coupled receptor. Recently, a novel PTH-like peptide (PTH-L), previously identified in fish, was found to be expressed in chicken, together with several alternative transcripts for PTHrP. *In vitro* tests reveal that all three PTH-family members stimulate calcium fluxes in chicken chorionallantois membrane and *Xenopus* abdominal skin. Although, their function role and tissue expression during embryogenesis still poorly documented.

Birds have characteristics which indicate a complex system in calcium regulation, iand chicken embryos are developmental models that can be easily manipulated experimentally. The present study investigated PTH-family expression during chicken embryogenesis. We have characterised this family of peptides in chicken by mapping gene expression with RT-PCR, whole mount *in situ* hybridization and 3D image reconstruction using Optical Projection Tomography microscopy.

PTH-L is expressed from at least 19 hours of incubation (stage 4HH), and is present during central nervous system formation (stage 11HH). Chicken PTH seems to be expressed along the parathyroids formation, being present in the four parathyroid glands; however, it is expressed also in the limbs (stage 29HH). The *PTHrP* alternative splices isoforms reveal specific tissue distributions during chicken embryogenesis, at least from stage 4HH. Moreover, *PTHrP* is present in wings during bone formation at stage 36HH.

The three parathyroid hormone family members are present during chicken embryogenesis suggesting an important role in the development. PTHrP isoforms are expressed in most stages and tissues, including classical calcium metabolizing tissues such as bone and intestine, which is congruent with its paracrine nature. It is hypothesized that PTH-L has a role during central nervous system formation and *PTH* may have different functions besides calciotropic activity.

4.1. Introduction

The parathyroid hormone (PTH) is an important endocrine factor secreted by the PTGs which was first discovered in 1925, but only isolated in 1950s and chemically characterised in 1970s from the bovine PTGs (Potts 2005). In vertebrates, this hormone is a potent calciotropic factor and promotes calcium homeostasis by mobilization of calcium from bones counteracting calcitonin. Fishes, including teleost, are an exception among vertebrates by not having PTGs and by having two *PTH* genes which express widely in different tissues (Danks, Ho et al. 2003; Potts 2005; Guerreiro, Renfro et al. 2007). Its calciotropic action resides in the 1-34 N-terminal amino acids region of the mature peptide (Gardella and Juppner 2001) which has high sequence homology to PTH-related peptide (PTHrP) and both hormones have the ability to activate the same receptor type (PTH1R) (Philbrick, Wysolmerski et al. 1996; Clemens, Cormier et al. 2001). PTHrP was cloned from human lung cancer tissue in 1987 (Moseley, Kubota et al. 1987) and together with its calciotropic importance, it is a paracrine/autocrine factor with a broad range of biological activities which correlate with its expression in many tissues (Clemens, Cormier et al. 2001; Guerreiro, Fuentes et al. 2001; Ingleton 2002; Martin 2005). Furthermore, a novel member of this family, the PTH-Like peptide (PTH-L), first reported in teleosts (Canario, Rotllant et al. 2006) is also present in *Xenopus* and chicken with conserved sequence, gene structure and calcium mobilization activity (Pinheiro, Cardoso et al. 2010).

In mammals, PTH has an important role in bone formation, turnover and skeletal development during adult life, acting mainly in bone and kidney (Swarthout, D'Alonzo et al. 2002; Potts 2005). However, non-classical target cells activity such as in erythrocytes, lymphocytes, liver cells, smooth muscle cells, pace maker and muscle cells of heart are also reported (Bro and Olgaard 1997; Schluter 1999) and its role during embryogenesis is not very clear. PTH is expressed in placenta and suggested to play a critical role in regulating foetal calcium homeostasis by promoting skeletal mineralization during development (Simmonds, Karsenty et al. 2010). In fish larvae, PTH expression was detected in the lateral line, central nervous system and jaw, although it seems to be absent in cells that are likely to correspond to a pharyngeal-derived PTG equivalent or in the thymus, suggesting a different role from calciotropic activity (Danks, Ho et al. 2003; Hogan, Danks et al. 2005).The PTHrP, is encoded by a single gene and due to posttranslational cleavage sites its precursor can originate three different mature fragments: the N-terminal region (amino acids 1-36) highly similar to PTH, the mid-region (amino acids 38-94) and C-terminal (amino acids 107-193) (Mangin, Ikeda et al. 1989; Orloff, Reddy et al. 1994; Power, Ingleton et al. 2000; Guerreiro, Renfro et al. 2007). Apart from calciotropic role, the different fragments are also involved in different functions and

are characterised by a dispersed tissue distribution being present in skin, mammary gland, smooth muscle, bone, cartilage, heart, lung, kidney, liver, central nervous system, placenta, amnion and prostate gland (Philbrick, Wysolmerski et al. 1996). During embryonic and foetal development, PTHrP has vital roles (Ingleton and Danks 1996; Wysolmerski and Stewart 1998) since it was found to be expressed in every embryonic tissue analysed (Campos, Asa et al. 1991; Schermer, Chan et al. 1991). In adition, PTHrP-knockout mice die at birth due to chondrodystrophic phenotype characterized by premature chondrocyte differentiation and bone formation (Philbrick, Dreyer et al. 1998) and ~907 kb genomic microdeletion on chromosome 12p affecting PTHrP gene and point mutations within PTHrP are associated with autosomal-dominant brachydactyly type-E (Klopocki, Hennig et al. 2010). Moreover, PTHrP is increased in the fetal circulation during late gestation and was hypothesis that might assume the actions of PTH during fetal life (Simmonds, Karsenty et al. 2010). On the other hand, serum PTH was increased threefold in PTHrP null foetuses, preventing more severe hypocalcemia (Kovacs, Chafe et al. 2001), however, PTH could not fully compensate the PTHrP absence (Simmonds, Karsenty et al. 2010). As in tetrapods, piscine PTHrP has a widespread tissue expression, present in gut, skin, gills, brain, pituitary and kidney with a range of different paracrine activity (Guerreiro, Renfro et al. 2007).

PTH-L is the most recent member of the PTH-family and few studies concerning its putative physiological role exist. Experiments using fish larvae indicate that similarly to PTHrP it is able to increase whole body calcium influx (Canario, Rotllant et al. 2006; Guerreiro, Renfro et al. 2007). There is also evidence that it can stimulate calcium flux in *Xenopus* and chicken epithelia (Pinheiro, Cardoso et al. 2010).

In chicken, the model species used in this study, there are single copy genes for PTH, PTHrP and PTH-L (Pinheiro, Cardoso et al. 2010). The PTH reveals share a role in calcium fluxes where is involved in regulation of cartilage growth, chondrocyte apoptosis, deposition and expression of type X collagen (Harrington, Lunsford et al. 2004) and to activate both Na^+/H^+-exchanger and Cl^--channel in proximal tubule (Laverty, McWilliams et al. 2003). Moreover, PTH was reported to affect the eggshell calcium percentage and breaking strength (Jiang, Xie et al. 2010) and stimulates calcium fluxes in the chorionallantois membrane, along PTHrP and PTH-L, from apical to basolateral region (Pinheiro, Cardoso et al. 2010). PTHrP was suggested to has a paracrine role for epiphyseal chondrocyte, regulating growth plate chondrocyte differentiation (Farquharson, Jefferies et al. 2001). Thus supporting that PTHrP is produced by the epiphyseal chondrocytes, limiting the rate of growth plate chondrocyte terminal differentiation (Pateder, Rosier et al. 2000). Moreover, five multiple transcripts have been identified for PTHrP in adult chicken tissues which give rise to two distinct mature PTHrP peptides of

139 and 141 amino acids suggesting a complex regulation system (Pinheiro, Cardoso et al. 2010), however, their distribution and function during ontogeny still unknown. The novel PTH-L also remains to be characterized in embryogenesis, which along the poorly understood foetal PTH rise questions about ontogenic gene expression and function of PTH-family members.

Chicken embryos have been vastly used as embryonic developmental model to study vertebrate gene function, since their embryos come conveniently packaged in calcified eggshells and can be easily manipulated experimentally (Brown, Hubbard et al. 2003). The present study aims to contribute to the understanding of PTH-family members in vertebrate development by characterizing their tissue distribution during distinct chicken embryogenesis by RT-PCR and whole-mount *in situ* hybridization (ISH) and 3D image reconstruction using Optical Projection Tomography.

4.2. Methods

4.2.1. Animals and tissue collection

White Leghorn chicken (*Gallus gallus*) eggs were kept at 37.5º C under high humidity conditions in an automatic incubator (Brinseca OCTAGON 40) with gentle rotation. Tissues for expression studies were collected and immediately frozen in liquid nitrogen and stored at -80ºC until used. The eggs used in *in situ* hybridization were windowed; the embryos removed to ice-cold phosphate buffered saline (PBS) (0.02M phosphate, 0.15M NaCl) and cleaned of extra-embryonic membranes. Eyes and forebrain were punctured to reduce trapping, and transferred overnight to 4% ice-cold paraformaldehyde (PFA). The embryos were then put through a graded methanol series at 4^{o}C; ending in 100% methanol washes and stored at -20^{o}C until use. All animal experiments were performed in accordance with Portuguese legislation under a “Group-1” licence from the Direcção-Geral de Veterinária, Ministério da Agricultura, do Desenvolvimento Rural e das Pescas, Portugal.

4.2.2. RNA extractions and gene expression

Total RNA was extracted from embryos at stages 4HH; 17HH; 26HH; 31HH (divided in head and body); 34HH (divided in head and body); 39HH (brain, intestine, liver and legs) and 44HH (chorionallantois membrane) (Hamburger and Hamilton 1992) using Tri Reagent (Sigma Aldrich, Spain) according to the manufacturer’s instructions. To avoid genomic contamination, RNA was previously treated with 1U DNase (DNA-free Kit, Ambion, UK) for 30 min at 37ºC. DNase treated RNA (500ng) was denatured at 65ºC for 5

min, quenched on ice for 5 min and used for cDNA synthesis in a 20 μl reaction volume containing 10 ng of pd(N)6 random hexamers (GE Healthcare, UK), 2mM dNTPs, 100U of MMLV-RT and 20U RNasin® Plus RNase inhibitor. cDNA was synthesized for 10 min at 20ºC followed by 50 min at 42ºC and 72ºC for 5 min. PCR reactions were performed using specific primers in the different regions of each transcript (Table 8). PTH primers are within the coding region, PTHrP primers are specific for each 5'UTR (Pinheiro, Cardoso et al. 2010) and PTH-L primers are in 5'UTR (forward) and coding region (reverse). The same sequences were used for *in situ* probe synthesis for PTH and PTH-L, whereas the PTHrP probe was within the coding region in order to be common to all transcripts.

Amplification reactions were carried out to a final volume of 25 μl with 1.5 mM $MgCl_2$ (Biocat, Germany), 0.2 mM dNTP's (GE Healthcare, Spain), 0.25μM of specific primers and 0.5 U of EuroTaq DNA Polymerase (5 U/μl, Euroclone, Italy) using in each cycle: 3 min at 94 ºC; 45 s at 94 ºC; 45 s at "specific primers annealing temperature ºC"; 1 min at 72 ºC cycled 35 times for PTH and PTHrP and 40 times for PTH-L; with a final extension of 10 min at 72 ºC. The annealing temperatures were 54 ºC for PTH, 55 ºC for PTHrP(1-139), 56 ºC for PTHrP(1-141), 54 ºC for PTH-L and 58 ºC for Gcm-2.

Table 8: Primer pairs used to amplify chicken PTH, PTHrP, PTH-L and Gcm-2 probes for *in situ* hybridization and for semi-quantitative analysis of gene expression (RT-PCR).

Probes	RT-PCR
PTHfw: atgacttctacaaaaaatctg *PTHrv: tggcttagttttaaagagta*	*PTHfw: atgacttctacaaaaaatctg* *PTHrv: tggcttagttttaaagagta*
PTHrPfw: agtggagtttcgcggtgttt *PTHrPrv: ggtatctgccctcatcttca*	*PTHrP(1-139)fw: ctgagagcccagtcttgga* *PTHrP(1-139)rv: gggtaacaatttcagtaact*
PTH-Lfw: gaacgacaagagaaggaaag *PTH-Lrv: ctgcttcatcgggtttga*	*PTHrP(1-141)5utrAfw: gaagggagtagcacctgggc* *PTHrP(1-141)5utrBfw: ggcacctgcttttaaaaccc* *PTHrP(1-141)5utrCfw: gctaacagaggaactgcgac*
Gcm-2fw: ctatcccgtcaccaacttc *Gcm-2rv:tcgctcctgtctccactgcc*	*PTHrP(1-141)5utrDfw: aggactgacccctcctttcc* *PTHrP(1-141)rv: gatcccctctactgatcttcc*
	PTH-Lfw: gaacgacaagagaaggaaag *PTH-Lrv: ctgcttcatcgggtttga*
	18Sfw: tcaagaacgaaagtcggagg *18Srv: ggacatctaagggcatcaca*

In order to confirm the quality and quantity of cDNA in the PCR reactions, 18S ribosomal RNA was also amplified (primers in Table 8) and served as an internal standard. The cycling protocol was similar to the target genes except for the annealing temperature which was 55 ºC and the 22 cycles. The PCR reaction products obtained were analysed on 1% agarose gels and sequenced to confirm identity.

4.2.3. *In situ* probe synthesis

The PTH-family members and Glial cells missing-2 (Gcm-2), a transcription factor expressed in the PTH-secreting cells of the PTG (Gunther, Chen et al. 2000)) were amplified by RT-PCR using specific primers (Table 8) and cloned into pGEM-T Easy vector. Plasmid DNA fragments were amplified using standard M13 primers (*Fw:* gtaaaacgacggccagtg; *Rv* ggaaacagctatgaccatg) and the PCR product obtained precipitated by the addition of acetate/ethanol following standard procedures (Maniatis, Fritsch et al. 1982). After cooling to -20^{o}C for <1hr the precipitated cleaned DNA fragments were collected in a micro centrifuge for 20 mins, washed in 80% ethanol and air-dried. The digested fragments were then resuspended in 10 µl of TE (10mM Tris-HCl pH 7.0; 1mM EDTA) and an aliquot quantified by running on an 1% agarose gel.

The PTH-family members RNA probes were prepared in reactions containing T7/SP6 RNA polymerase enzyme buffer; 100mM DTT, Dig-NTP mix (Roche), 100U RNase inhibitor (Roche), 1µg linear DNA fragments and 10U of the appropriate RNA polymerase (Roche). After, incubation at 37^{o}C for 1hr, 1 µl of the reaction was run on agarose gel to quantify the RNA product. The probe was purified using the ProbeQuant G-50 spin column system (Amersham Biosciences) and used in the *in situ* procedures.

4.2.4. *In situ* hybridization

In situ hybridizations (ISH) were performed using a modification of the method proposed by Nieto et al. (1996) for whole mount and tissue sections. Prior to probe incubations, chicken embryos and sections were rehydrated through a graded series of 4 ^{o}C MeOH (EtOH in tissue sections), washed in 100% PBT and treated with 20 µg/ml proteinase K in PBT for up to 20 min at room temperature and rinsed 3 times in PBT before being placed into pre-warmed PBT and incubated at 65 ^{o}C for 30 min. Embryos were transferred to hybridization buffer at 65 ^{o}C and pre-hybridized for at least 1 hr. Approximately 1ng of probe was added per µl of fresh hybridization solution and chicken embryos were incubated for 16 hr at 65^{o}C and washed for 10 min in fresh hybridization buffer at 65 ºC using a series of different stringency SSC/CHAPS solution as follows: 2 washes of 10 min in 2xSSC, followed by 3 washes of 20 min in 2xSSC/0.1% CHAPS and 3 washes of 20 min in 0.2xSSC/0.1% CHAPS to remove background signal. Tissue sections were washed in 2xSSC and 3x30 min in 50 %formamide/2xSSC at 65 ºC. Embryos and tissue sections were washed twice in MABT (maleic acid buffer/0.1% TWEEN20) at room temperature for 10 min and immersed in blocking buffer (3% Boehringer Blocking Reagent (BBR) in MABT) for 2-3 hr prior to overnight incubation with a 1:1000 dilution of anti-Dig antibody (Roche) in fresh blocking buffer at 4 ^{o}C. A total of 5

washes in MABT were performed and washing solution was changed every hour complemented with a final overnight wash at 4ºC to ensure complete removal of any non-specific antibody hybridization. On the following day, embryos and tissues were pre-incubated twice for 20 min at room temperature in freshly made NTMT (100 mM NaCl, 100 mM TrisHCl pH 9.5, 50 mM MgCl2, 0.1% TWEEN 20) and subsequently stained with BCIP (175 µg/ml) and NBT (350 µg/ml) in NTMT. This reaction was carried out at 4 ºC or at RT in a light proof container and the coloured reaction was stopped by the addition of 4% formal saline solution at 4 ^{o}C.

4.2.5. Optical Projection Tomography (OPT) Scanning and 3D mapping

The ISH positively stained embryos were fixed in 4% PFA for 30 min and washed 3 times for 20 min in PBS followed by two washes for 10 min in distilled water, left overnight in distilled water followed by a 10 min wash with fresh distilled water in the following day. Before attachment to the OPT scanner, embryos were embedded in 1% low melting point agarose and immersed in BABB (1:2 benzyl alcohol to benzyl benzoate) for at least 4 hr. OPT scanning was carried out following the protocol described by Sharpe et al. (2002). An autofluorescence scan of the sample was first carried out and was followed by a bright-field scan of the signal in the same sample. The scan consisted of 400 digital photos taken through the embryo during a 360^{o} rotation, detecting and recording any object interrupting the light path on the way. The 3D mapping was performed using the Amira 4.1 software from Mercury Computer Systems.

4.3. Results

4.3.1. RT-PCR expression of the chicken PTH-family members

The tissue distribution of chicken PTH-like transcripts was initially characterised in chicken embryos by RT-PCR with specific primers to amplify the three PTH-family members and the PTHrP alternative splice forms. The objective was a) to have a general profile of their distribution which could suggest a putative role during chick development, associated with the formation of calcified structures and b) to identify the tissue and stages in which to perform *in situ* hybridization (Figure 29).

PTH-family members are expressed from early stages of development with PTHrP showing the widest distribution pattern. This expression pattern contrast to PTH which demonstrates a restricted distribution, whereas PTH-L is mostly expressed in early development stages. PTH expression was only detected after 5 days (26HH), which corresponds to the stage of development of elbow and knee joints, the first three toes and

beak formation and the PTGs appear. At stage 31HH, PTH expression was detected in the body section, which includes the PTG region. However, at 34HH, due to body growth the signal ratio PTH mRNA / 18S RNA decreases. With the exception of PTH, all PTH-like members were found to be expressed in chicken limbs of 13 day embryos (39HH). Moreover, the isoforms ckPTHrP(1-141)5utrC and ckPTHrP(1-39) appear to be the most widely expressed of the PTHrP isoforms. ckPTHrP(1-141)*5utrA* seems to be mainly present up to 31HH, and some expression was also detected in the legs of 39HH embryos. The expression of ckPTHrP(1-141)*5utrB* seems to initiate at the same time as PTH and after the 31HH stage it appears mostly in the legs as with the other isoforms. PTHrP(1-141)*5utrD* seems to be the less expressed isoform, with a pattern which resemble PTH-L. The 44HH chorionallantois membrane expresses PTHrP(1-141)*5utrC,* PTHrP(1-141)*5utrD* and PTH-L. PTH-L was found to be mainly expressed at 19 hour (4HH) (definitive primitive streak formation) and in liver of 13 day (39HH) chicken embryos, although PTH-L also appear faintly at other stages (Figure 29).

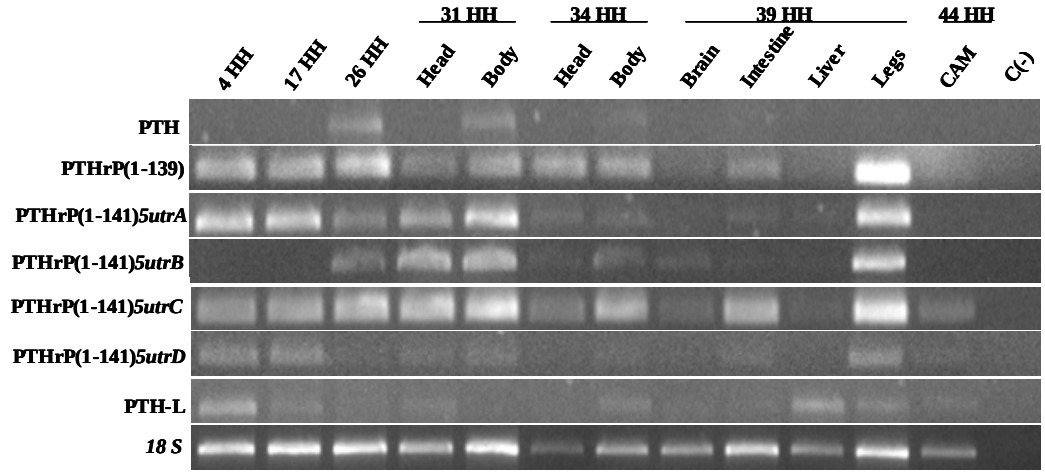


Figure 29: PCR expression of PTH-family members during chicken embryonic development (4 embryos pool of each stage: 4HH, 17HH, 26HH, 31HH, 34HH, 39HH, 44HH). $C^{(-)}$ represents the negative control reaction. 18S ribosomal amplification control is shown for each tissue.

4.3.2. In situ hybridization (ISH)

Whole mount *in situ* hybridization was carried out to analyse expression localization of the PTH family of genes. As suggested from RT-PCR, PTH starts to be expressed around stage 25HH (approximately 5 days of incubation) in the PTGs (Figure 30a1) and expression is maintained through stages 27HH and 28HH. To confirm the PTG localization, the Gcm-2 transcription factor expression, specific for the mammalian PTG, was carried out and an intensive staining was obtained in the same region as PTH (Figure 30b1-2b3).

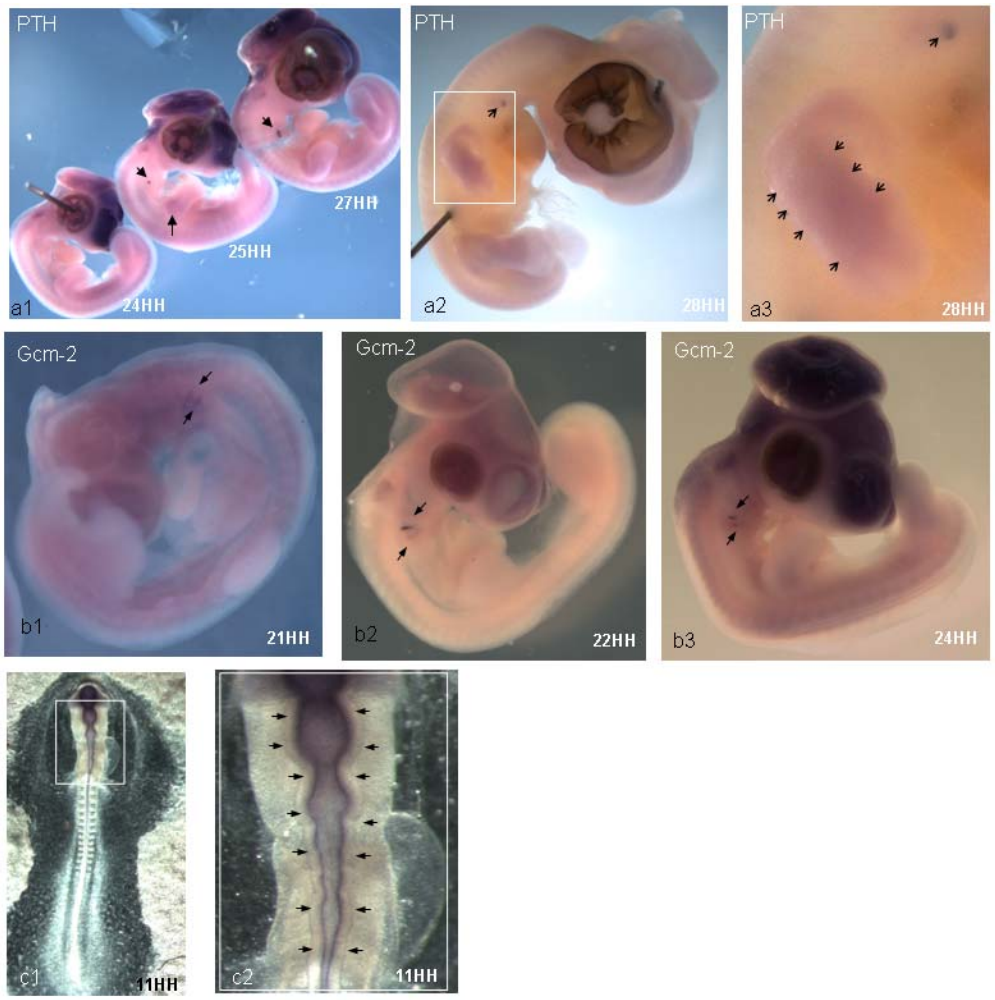


Figure 30: Gene expression of (a) PTH, (b) Gcm-2 and (c) PTH-L in chicken embryos at different developmental stages detected by whole mount in situ hybridization. Specific signal detection is indicated by arrows and the white square in a2 and c1 is detailed in a3 and c2, respectively. The developmental stages are given in each photograph.

Moreover, a PTH weak hybridization signal was also detected in the 28HH chicken limbs (Figure 30a2-3) which was supported by RT- PCR (Figure 31) revealing the presence of PTH transcript in trunks with PTG, but also in the limbs and in the head (where the beak is in formation).

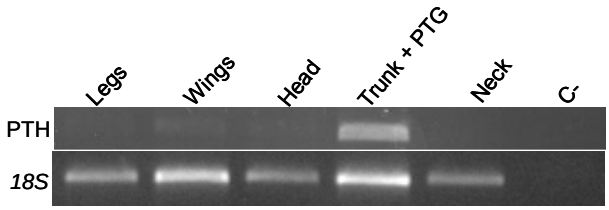


Figure 31: Distribution of the chicken PTH by RT-PCR at different body sections at stage 29HH. 18S amplification control is shown for each tissue.

The 3-D image reconstruction using the OPT data from ISH shows high PTH expression in the four PTGs of the embryos at stage 26/27HH (Figure 32a1-3), but it was not possible to confirm PTH expression in limbs or head with this technique.

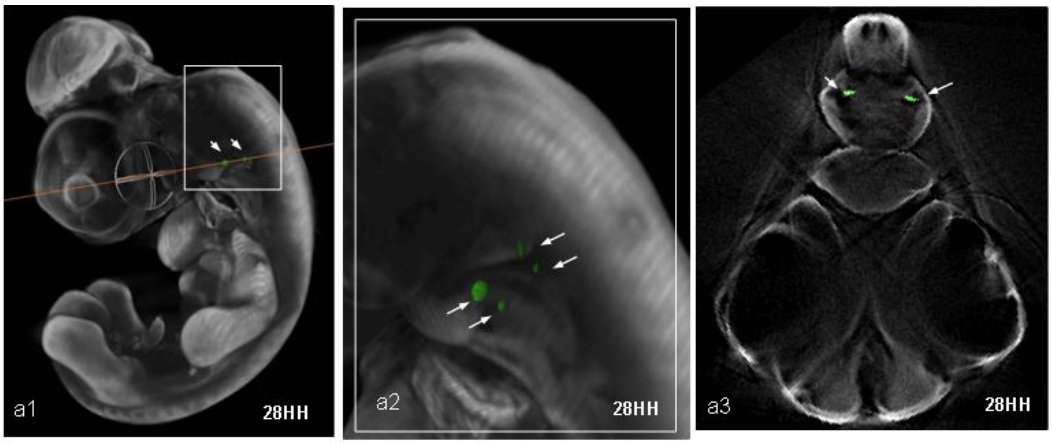


Figure 32: 3D mapping using embryos from ISH in the OPT showing PTH gene expression in the PTGs given by the green colour and indicated by arrows. “a1” shows the general view. “a2” is a detailed view of the region represented by a white box in “a1” showing the four PTGs. “a3” is the cross-section represented by the orange line in “a1”.

Since several probes were tested with similar difficulty to detect specific PTHrP and PTH-L expression using whole mount ISH, this may reflect their dispersed and low intensity of tissue distribution as observed by RT-PCR and reported others (Schermer, Chan et al. 1991; Pinheiro, Cardoso et al. 2010).

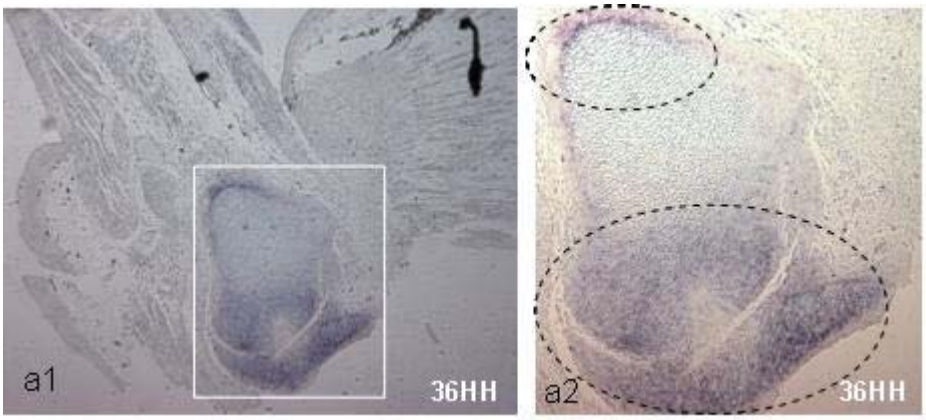


Figure 33: ISH expression of PTHrP in a wing section. “a1” is general view of the section of a wing from stage 36HH. The detailed view of expression region represented in “a1” by a white box is represented in “a2”. Dotted circles indicate the region with most expression PTHrP containing chondrocytes in proliferation/differentiation stages.

The PTHrP and PTH-L expression originated a diffuse stain in whole mount ISH at these stages and therefore it is not possible the identification of a specific tissue localization. However, staining was detected in chicken wings sections for PTHrP and nervous system for PTH-L in early stages. Expression of PTHrP was observed in the wings cartilage/bone sections at 10 days of incubation (36HH) (Figure 33a1-2) which may be in agreement with PTHrP function in switching proliferating to differentiating chondrocytes (Minina, Kreschel et al. 2002). PTH-L expression was detected only in early stages of development in the nervous system of 10HH (~40 hours) embryos (Figure 30c1-2).

4.4. Discussion

Until recently, only two hormones, the PTH and PTHrP, were known to be present in tetrapods. However, the discovery in chicken of a homologue of fish PTH-L suggests its involvement in yet unidentified functions. Furthermore, the presence of several distinct PTHrP transcripts, in chicken resembling the situation in mammals, indicates the existence of a complex regulatory system which is far from being understood. This study demonstrates that all members of the PTH-family are expressed from very early stages during chicken development and each gene has a specific pattern. *PTHrP* has the most widespread expression pattern with common features to mammalian expression. *PTH* is not only expressed in the PTG but also in limbs. *PTH-L* is mainly expressed in early embryonic stages during nervous system formation, suggesting a specific role in this process.

PTH expression starts at 25/26HH developmental stage, as in mammals, in which expression of PTH is mainly in the PTGs. However *in situ* hybridization localized expression to the chicken wings in the 28HH stage, which could be associated with the formation of cartilage/bony structures. In adult chicken, PTH expression is not exclusive to the PTG and was also noted in the pituitary, bone and cartilage (Pinheiro, Cardoso et al. 2010). Moreover, in mammals, PTH is also secreted in non-PTG tissues as the brain/pituitary (Balabanova, Peter et al. 1986; Pang, Harvey et al. 1988) and in case of genetic PTGs ablation, PTH is also expressed in thymus (Gunther, Chen et al. 2000). There is report of ectopic expression in placenta (Balabanova, Grosseloh et al. 1986; Simmonds, Karsenty et al. 2010) in some patients with cancer, attributed to molecular rearrangements of the PTH gene (Suva 2006; Demura, Yoneda et al. 2010). In fishes, which do not possess a PTG, PTH was also found to be expressed in different tissues such as pituitary, brain (Harvey, Zeng et al. 1987), nervous system and lateral line

(Danks, Ho et al. 2003; Hogan, Danks et al. 2005). These findings suggest that PTH expression became more restricted to a gland during terrestrial adaptation, which appear as a separate endocrine gland in amphibians, to control free calcium levels, detected by the calcium-sensing receptor (CaSR) (Zajac and Danks 2008). Several different specific factors (Tbx1, Hoxa3, Pax1, Pax9, Eya1, Sp1, Sp3, NFYα, NFYβ, NFYγ and Gcm-2) (Maret, Bourdeau et al. 2004; Alimov, Park-Sarge et al. 2005) play a critical role in the development of the normal PTG and modulate PTH secretion. Among them the transcription factor Gcm-2 is essential for PTG formation (Liu, Yu et al. 2007) and it was found to be expressed in the 3rd and 4th pharyngeal pouches (Figure 30b) where PTG arises (Okabe and Graham 2004). However, it has been reported that it is not PTG exclusive, being expressed in other tissues in small amounts such as intestine, kidney, gall bladder, liver, pancreas (Correa, Akerstrom et al. 2002). Moreover, Gcm-2 continues to be expressed and active in mature PTGs regulating CaSR expression (Mizobuchi, Ritter et al. 2009). CaSR also has a widespread expression, occurring not only in the thyroid and PTGs, but also in kidney, bone, cardiovascular system, liver, gastrointestinal tract, and central nervous system (Armato 2009; Hendy, Guarnieri et al. 2009; Nanjo, Nagai et al. 2010). This overlapping of Gcm-2, PTH and CaSR in tissues other than the parathyroid, could suggest that the PTH expression modulation mechanism extends beyond PTG, with involvement in different functions in other tissues.

PTHrP is expressed in all tested embryonic tissues, however, the different isoforms expression profiles suggests they may have distinct functions at different development stages (Pinheiro, Cardoso et al. 2010). The PTHrP isoforms are expressed in cartilage of legs stage 39HH (Figure 29) and wings 36HH (Figure 33) confirming their important role in this tissue during embryogenesis (Farquharson, Jefferies et al. 2001). The ckPTHrP(1-141)5utrC and ckPTHrP(1-39), which appear to be the most widely expressed isoforms, suggest to be the transcripts related to a conserved PTHrP role during embryogenesis. Their expression in tissues such as intestine and legs could indicate a conserved role in calcium metabolism. In contrast, ckPTHrP(1-141)*5utrA* and PTHrP(1-141)*5utrB* seems to be involved in specific activity, which remain to be identified, in different developmental stages. The PTHrP and PTH-L tissue expression overlaps in the majority of the tissues and stages analysed suggesting widespread paracrine actions for the two proteins as described for adults (Pinheiro, Cardoso et al. 2010).

Despite PTH-L overlapping expression with PTHrP transcripts, it has a diffuse expression being mainly present in early stages (Figure 29), where it seems to be present during formation of the central nervous system, (Figure 30c) and is in agreement with its expression in chicken and *Xenopus* brain (Pinheiro, Cardoso et al. 2010). However, at present there is no information to suggest what could be its possible role. We have

previously shown it can stimulate calcium fluxes (Pinheiro, Cardoso et al. 2010) but it cannot be ruled out a function as a neurotransmitter or neuromodulator.

Probably due to *PTHrP* widely expression, the probes for a common region of all *PTHrP* transcripts were not able to indicate a specific tissue signal using whole mount embryos and more specific probes are needed to test their expression.

In conclusion, all parathyroid hormone family members are expressed during chicken embryogenesis. *PTH* is expressed not only in the PTGs but also in limbs. *PTHrP* isoforms are expressed in most tissues, including classical calcium metabolizing tissues such as bone and intestine and *PTH-L* is expressed early during central nervous system formation. Further studies are needed to confirm the hypothesis that PTH may have different functions during embryogenesis and to explore the role of PTH-L in the central nervous system.

CHAPTER V

Abnormal Bone Development After Chicken PTHrP and PTH-L Knock-down

With: Mathews Towers, Fiona Bangs, Cheryll Tickle, Deborah M. Power & Adelino V.M. Canário

Abstract

The parathyroid hormone (PTH) and PTH-related protein (PTHrP) are the two principal calciotropic endocrine factors that play a fundamental role in body calcium homeostasis, bone formation and skeletal development. However, PTHrP also plays autocrine/paracrine roles in a range of different tissues, and its calciotropic action is bring about by the activation of the PTH receptor, a seven transmembrane receptor of family 2 G-Protein Coupled Receptors (GPCRs) B1. Another member of this family, PTH-Like peptide (PTH-L) has been identified in fishes, amphibian and birds, although it appears to be absent in placental mammals. *In vitro* tests reveal that all three PTH-family members stimulate calcium fluxes in chicken chorionallantois membrane and *Xenopus* abdominal skin. PTH-family is expressed during embryogenesis, although, their embryonic function still poorly understood. This study investigated PTHrP and PTH-L role in skeletogenesis during chicken embryonic development.

PTHrP knock-down during wing development promotes the digit 3 bone development and decreases its cartilage length. A similar action occurs with PTH-L which knock-down decreases cartilage length in scapula; however no alterations were observed in bone.

PTHrP and PTH-L have a potential role in cartilage and bone development during chicken embryogenesis. PTHrP knock-down supports its anti-apoptotic action in chondrocytes decreasing cartilage length. The novel PTH-L seems to share to PTHrP a similar role in cartilage development suggesting a common pathway in this process.

5.1. Introduction

The internal skeletal is a vital calcium pool for vertebrates (Bentley 1998), in which hormones such as parathyroid hormone (PTH) and PTH-related protein (PTHrP) act has potent calcium mobilization factors involved in bone turnover and skeletal formation (Philbrick, Wysolmerski et al. 1996; Potts 2005; Bar 2008). Recently, a new member of this family, PTH-Like peptide (PTH-L), has been identified in teleost fish, *Xenopus* and chicken, and shown to be present in the genomes of other vertebrates, although it appears to be absent in placental mammals (Pinheiro, Cardoso et al. 2010). Members of this family binds and activate specific seven transmembrane receptors of family 2 G-Protein Coupled Receptors (GPCRs) B1 (Gensure, Gardella et al. 2005). Whilst in mammals two specific receptors, *PTH1R* and *PTH2R*, were identified, an extra third receptor (*PTH3R*) was also found in teleosts (Juppner, Abou-Samra et al. 1991; Rubin and Juppner 1999) and chicken, which possess two functional PTH receptors (PTH1R and PTH3R) but lack PTH2R.

Most investigations on the role of PTH-family members and their receptors on skeletal development were carried out using mammalian models; however, recent studies in chick found that PTH enhances calvaria gap junctional intercellular communication among osteocytes, which are more connected with bone formation than reabsorption (Ishihara, Kamioka et al. 2008). In mammals, PTH contribute to the developed of angiogenesis/capillary invasion, which in PTH-null mice lead to reduced cartilage matrix mineralization and reduced entry of precursors for cells of the osteoblastic and chondroclastic lineages, decreasing bone tissue length (Miao, He et al. 2002; Diamond, Gonterman et al. 2006; Rhee, Park et al. 2009). Moreover, PTH also seems to play a dominant role in regulating foetal blood calcium which is a determinant for skeletal mineral accretion during embryonic stages (Kovacs, Chafe et al. 2001). In contrast, PTHrP expression was insensitive, at least in growth plate cartilage of PTH null mice, to changes in plasma calcium concentration (Miao, He et al. 2002). In PTHrP ablation the major consequences occurs in the avascular cartilaginous growth plate, where PTHrP exert autocrine/paracrine actions (Amizuka, Warshawsky et al. 1994; Karaplis, Luz et al. 1994). This leads to an acceleration of differentiation of growth plate chondrocytes and an increase in osteoblast number and cortical bone (Lanske, Amling et al. 1999), which contrasts with overexpression studies in which inhibition of chondrocyte terminal differentiation is observed (Weir, Philbrick et al. 1996). In addition, silencing of PTH1R in mice causes abnormal endochondral bone formation, similar to the phenotype in PTHrP null mice (Lanske, Karaplis et al. 1996). However, the action of PTHrP in placental calcium transport (Kovacs, Chafe et al. 2001) was suggested to be mediated through a

receptor different from PTH1R (Kovacs, Lanske et al. 1996) and other receptors seem to be involved in morphogenesis, cell proliferation, apoptosis and calcium homeostasis mediated by PTHrP (Toribio, Brown et al. 2010) which remains to be identified.

The aim of this study is to contribute to the understanding of cell differentiation. During skeletogenesis in chicken embryos, a cartilaginous model is formed from mesenchymal condensations (Wright, Hargrave et al. 1995) and the process of chondrocyte differentiation culminates in calcification of the matrix and cellular apoptosis (Hatori, Klatte et al. 1995) and represents a good target to study PTH-family system function. Chicken embryos are a well-established developmental (Tickle and Eichele 1994; Tickle 2004; Towers and Tickle 2009) and genomic model system, which allows an introduction of DNA constructs by electroporation to perform a controlled gain or loss of function studies (Nakamura, Katahira et al. 2004). This study used reverse genetics techniques to investigate the role of PTH-family members and receptors in chick limb bone development. Overexpression of target cDNAs was carried out using a replication-competent avian retroviral vector *in vivo* (Cory and Adams 1988; Kubota, Hayashi et al. 2005) and knock-down assays used morpholino oligonucleotides (Tucker 2002).

5.2. Methods

5.2.1. RNA extractions and transcript amplification

White Leghorn chicken (*Gallus gallus*) eggs were kept at 37.5ºC under high humidity conditions in an automatic incubator. Whole embryos for RNA extraction were collected and immediately frozen in liquid nitrogen and stored at -80ºC until used.

Total RNA was extracted from stage 38HH (Hamburger and Hamilton 1992) using Tri Reagent (Sigma Aldrich, Spain) according to the manufacturer's instructions. To avoid genomic contaminations, RNA was treated using 1U DNase (DNA-free Kit, Ambion, UK) for 30 min at 37ºC. DNase treated RNA (500ng) was denatured at 65ºC for 5 min, quenched on ice for 5 min and used for cDNA synthesis in a 20 μl final reaction volume containing 10 ng of pd(N)6 random hexamers (GE Healthcare, UK), 2 mM dNTPs, 100 U of MMLV-RT and 20 U RNasin® Plus RNase inhibitor. cDNA was synthesized for 10 min at 20ºC followed by 50 min at 42ºC and 72ºC for 5 min. PCR reactions to amplify the chicken PTH-family members were performed using gene specific primers and longer modified primers with restriction sites for cloning into overexpression vectors (NcoI and EcoRI) (Table 9). Amplification reactions were carried out to a final volume of 25μl using 0.25μM of each primers, 0.5U of Advantage PCR polymerase (Clontech), 0.2mM dNTP's (GE Healthcare) using the thermocycle: 94ºC for 3min; cycled 35 times at 94ºC for 45s,

60ºC for 45s and 72ºC for 1min and final extension at 72ºC for 10min. To confirm the quality and quantity of cDNA utilized in each PCR reaction, 18S ribosomal RNA was amplified with chicken specific *18Sfw* tcaagaacgaaagtcggagg and *18Srv* ggacatctaagggcatcaca oligonucleotides and served as standard using to the same thermocycle as above except for the annealing temperature which was 55ºC. The PCR reaction products were analysed on 1% agarose gels and sequenced to confirm identity.

Table 9: Primer pairs used to amplify the coding region of chicken PTH-family ligands and receptors. Restriction sites are indicated at bold.

pSLAX13 cloning	T. ºC	pGEM-T Easy cloning	T. ºC
PTHrsfw: gactc**catgg**cttctacaaaaaatctgg *PTHrsrv:* gttc**gaattc**tcatggcttagttttaaagag	60	*PTH1Rfw:* atgggatcatatctggtttat *PTH1R:* ttacatcactgtctctctttc	57
PTHrPrs139fw: gactc**catgg**tgttcacgaaactctttcag *PTHrPrs139rv:* gttc**gaattc**ttaccttaaaatatgattatg		*PTH3R: atggggtctgtgggcagg* *PTH3R: tcatagcatcgtctccagct*	59
PTHrPrs141fw: gactc**catgg**tgttcacgaaactctttcag *PTHrPrs141rv:gttc**gaattc**tcagcgcctccttaaaatatg*			
PTH1Rrsfw: gactc**catgg**gatcatatctggtttatcac *PTH1Rrsrv:* gttc**gaattc**ttacatcactgtctctctttc			
PTH3Rrsfw: gactc**catgg**ggtctgtgggcagggg *PTH3Rrsrv:* gttc**gaattc**tcatagcatcgtctcc			

5.2.2. Construction of the overexpression of PTH/PTHRs vectors

In order to analyse the PTH-family ligands and receptors overexpression during chicken development, they were cloned into RCAS or pCAGGS vectors. pCAGGS vector has a chicken β-Actin Promoter which permits high expression levels (Kubota, Hayashi et al. 2005). However, shortly, as cells proliferate its expression becomes weaker. The RCAS [RCASBP(A)] (Hughes, Greenhouse et al. 1987) is a replication-competent avian-specific retroviral vector, which after being electroporated spreads into the cells and replicates during cell proliferation, and functional for longer periods (Hughes, Greenhouse et al. 1987; Cory and Adams 1988; Federspiel and Hughes 1997; Logan and Tabin 1998; Tam, Hughes et al. 2002). To proceed with these constructs, cDNAs should be cloned into the unique ClaI restriction sites of RCAS retroviral vector via an intermediate cloning step, using pSLAX13 shuttle vector which contains several restriction sites flanked by two ClaI restriction sites to facilitate cloning, which enhance the level of insert expression, and has the advantage of allowing cloning to be carried out in a high-copy number vector (Logan and Tabin 1998).

pSLAX13/RCAS was used to clone the ligand cDNAs and pCAGGS the receptors, and all three vectors were a gift of Dr Mathews Towers (University of Bath, UK). Control pCAGGS and RCAS vectors expressing red fluorescent protein (RFP) and green fluorescent protein (GFP), respectively, were provided by Dr Fiona Bangs (University of Bath, UK). Working solutions contained 1 µg of RCAS plus 1 µg of pCAGGS and 0.02% fast green, in a total volume of 5 µl.

The cDNAs of the coding regions of PTH-family members flanked by NcoI and EcoRI restriction sites and vector pSLAX13 were digested with NcoI and EcoRI (BioLabs, UK), according to the manufacturer's instructions. Ligation reactions were performed overnight at 4^0C for a final volume of 10 µl, containing 15 ng of vector, 50-100 ng of purified PCR product, 5µl of 2x rapid ligation buffer and 1.5 units of T4 DNA Ligase (BioLabs). Bacterial transformations were carried out using One Shot TOP10 Chemically Competent *E. coli* (Invitrogen) and 5µl of ligation product. Positive clones were selected and inoculated into 3ml LB broth with 80µg/ml ampicillin and grown overnight at 37^0C with shaking. Recombinant plasmids were extracted using QIAGEN Plasmid Maxi Kits (QIAGEN, UK), sequenced, and digested with ClaI (BioLabs) for cloning into RCAS vector. RCAS vector was linearized also with ClaI, dephosphorylated using Alkaline Phosphatase, Calf Intestinal (CIP) (BioLabs) and the digested products (Recombinant plasmid and RCAS vector) purified using the phenol/chloroform method (Maniatis, Fritsch et al. 1982) and ligated.

For the PTH-receptors the chicken PTH1R and PTH3R full length PCR products were cloned into pGEM-T Easy vector (Promega) and the recombinant vector was linearized using EcoRI (BioLAbs), purified using the standard phenol/chloroform method (Maniatis, Fritsch et al. 1982) and inserted into the pCAGGS vector EcoR I digested, dephosphorylated and purified as previously described.

5.2.3. Morpholino oligonucleotides

Independent morpholino antisense oligonucleotiude (MO) translation blockers for PTHrP (for PTHrP MO blocks the region common to all known isoforms identified) and PTH-L, were designed and synthesized by GeneTools (Gene Tools, LLC, USA) (Table 10). In order to check MO specificity, two negative controls, with 5-mismatches were also ordered for each MO. A standard oligo modification (3' fluorescein) was inserted in each morpholino to monitor fluorescence after microinjection. Due to the high price, only MO for PTHrP and PTH-L were acquired. The MO stocks were diluted to 4 mM in deionized miliQ water and working solutions were made at different concentration from 0.5 mM to 2 mM (Tucker 2002) in water and with 100 ng of RFP DNA inserted in pCAGGS, to aid in

electroporation and to check delivery, plus 0.02% fast green (Mende, Christophorou et al. 2008).

Table 10: Chicken morpholinos oligonucleotides sequences synthesised.

MO sequences
PTHrP: tttcgtgaacatcatatcctccggg
PTHrP*control*: ttacctgaagatcatatcctgccgg
PTH-L: gggcaggaacattttctctcctctt
PTH-L*control*: ggccacgaacatattctgtgctctt

5.2.4. Microinjection and electroporation procedures

Microinjection and electroporation (Figure 34) was carried out according to the methodology described by Tucker (Tucker 2002). Eggs containing white leghorn chicken embryos at stage 13HH were windowed, the embryos were highlighted with India ink (1/20) and approximately up to 0.2µl of work solutions were microinjected into the coelom. Microelectrodes (CUY21, Bex company, Tokyo) were then placed perpendicular to the long axis of the embryo on either side of the filled lumen and the solutions were electroporated into the wing form region with 5 pulses of 60 volts, 50 ms on, 50 ms off (CUY21, Bex company, Tokyo).

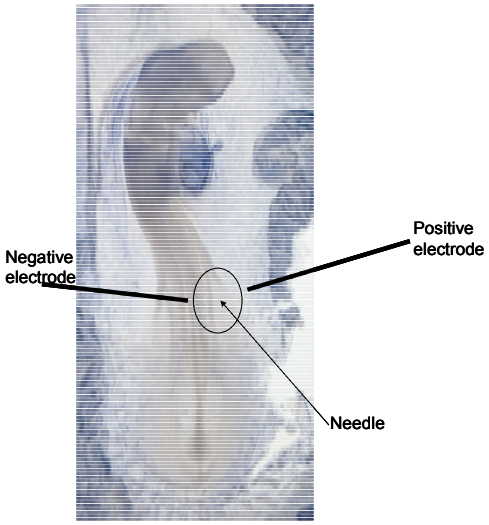


Figure 34: General representation of microinjection and electroporation procedures carried out during this study in chicken embryo coelom at stage 13HH. The positions of negative and positive electrodes used in electroporation are represented and the local of needle injection for the microinjection procedures is circled.

The window was then sealed with tape, and the embryos were returned to the incubator until day 10. The degree of RFP and GFP expression in the embryos was

observed under a microscope with UV light with texas-red (RFP) and GFP filters. The microinjection and electroporation was also tested following the same strategy directly in the limb at stage 22H.

5.2.5. Embryo skeletal staining

10 days after development, treated and control chicken embryos were collected and changes in bone and cartilage development were observed using Alcian Blue (cartilage) and Alizarin Red (bone) staining (Figure 35) following the published procedures (McLeod 1980). The embryo limbs, bone and cartilage from scapula, humerus, radius, ulna and digit 3 were measured in order to analyse growth alterations using the Open Lab programme 5.5 (PerkinElmer).

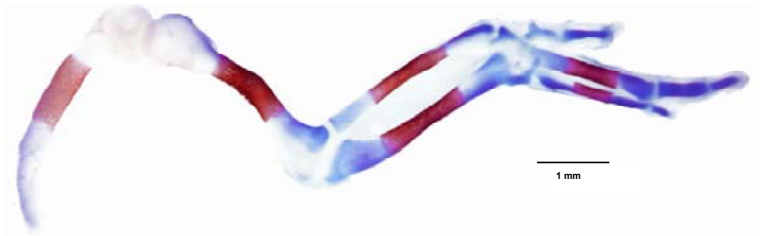


Figure 35: Alcian Blue and Alizarin Red staining of a right chicken limb at stage 36HH, from embryo electroporation control, RCAS+pCAGGS. Red represents the bone and blue the cartilage.

5.2.6. Statistical analyses

The results are presented as the mean ± SEM of six independent experiments for PTHrP and PTH-L. Paired t-test was used to identify statistically significant differences ($P < 0.05$) between wild type and morpholino's wings, using SigmaStat3.11 and the graphs were performed using the SigmaPlot9.01 programme.

5.3. Results

5.3.1. Construction of the PTH-family members overexpression vectors

RT-PCR using specific primers to amplify PTH-family members (PTH, PTHrP139 and PTHrP141 isoforms) and receptors (PTH1R and PTH3R) were confirmed by sequencing. Moreover, the chicken PTH, PTHrP139 and PTHrP141 were successfully cloned into pSLAX13 for posterior insertion into RCAS vector. PTH1R and PTH3R were cloned in pGEM-T Easy vectors (due to their length to pSLAX13) for posterior insertion into pCAGGS vector. Unfortunately, all the cloning strategies using the dephosphorylation

of pCAGGS and RCAS vectors digested products failed and overexpression analysis of the PTH-family members could not be performed. The reason for this could be associated with a technical problem that may had involved *ClaI* digestion or vector dephosphorylation reactions and need to be repeated in the future. However, to test the used methodology and to access if this procedure could be implemented in a near future, microinjections with the control tests using RFP and GFP cloned into pCAGGS and RCAS vectors were performed. Microinjections and electroporation with the control vectors were successful (Figure 36) since both GFP and RFP expression was possible to observe 3 days after electroporation. There was limited expression of pCAGGS+RFP, 8 days after electroporation, in comparison with RCAS+GFP expression (Figure 36). Thus RCAS seems a better vector to use when longer expression of transcript is required.

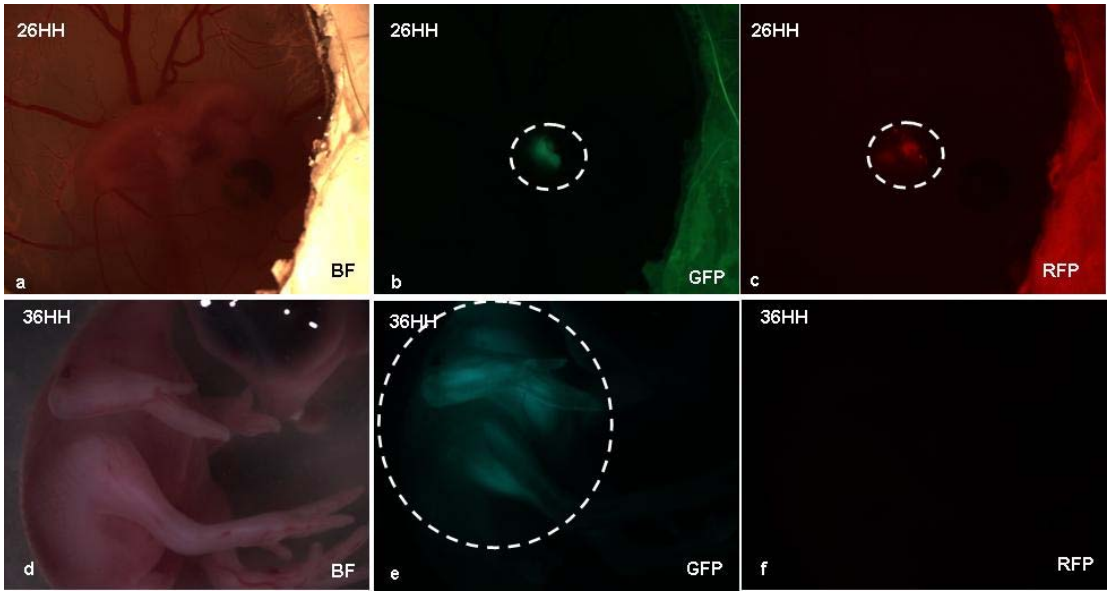


Figure 36: Chicken embryos with microinjected and electroporated with pCAGGs(RFP) and RCAS(GFP). The electroporation was carried out in the wing region at stage 13HH and the figure shows GFP and RFP expression within dotted white circle at stages 26HH and 36HH. BF: brighter field, GFP: GFP filter, RFP: texas-red filter.

5.3.2. PTHrP and PTH-L knock-downs

The potential roles of PTHrP and PTH-L on chicken wing skeletogenesis was investigation through a knock-down approach using microinjection and electroporation of specific morpholinos oligos (MO) in 13HH coelom (wing form region). At the moment of electroporation it was possible to observe the working solution in the coelom (Figure 37b), and as expected no RFP expression (Figure 37c). Approximately 24 hours later, it was

possible to observe some spread of MO which was not electroporated (Figure 37e). However, successful delivery was achieved as indicated by RFP expression (Figure 37f).

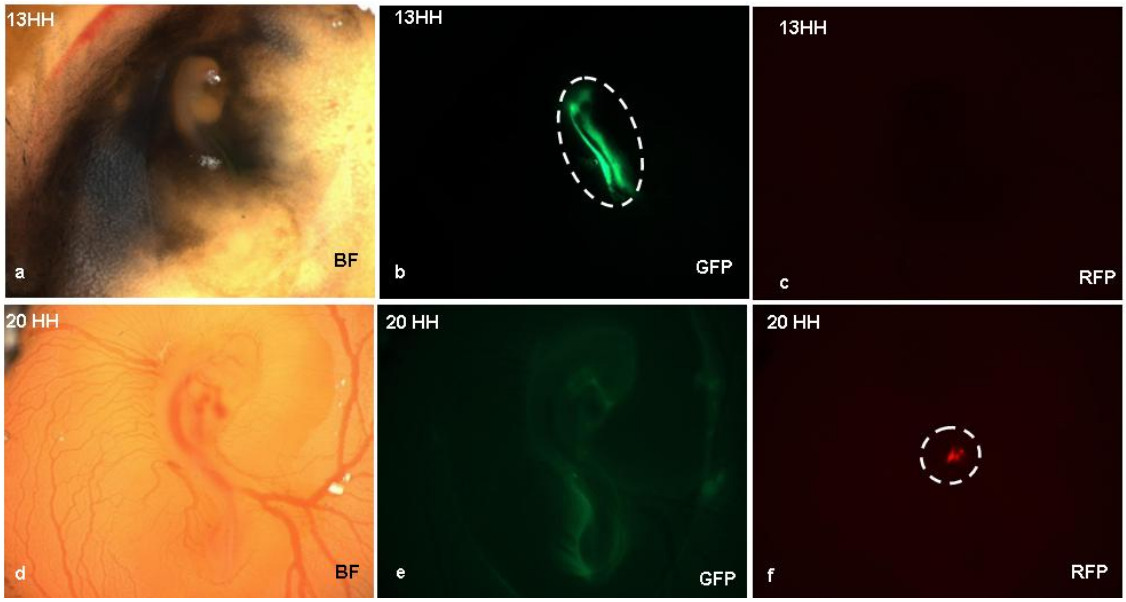


Figure 37: Chicken embryo microinjected and electroporated with MO. From "a" to "c", stage 13HH, from "d" to "f", stage 20HH using different microscope filters to observe GFP and RFP expression. BF: bright field, GFP: GFP filter, RFP: texas-red filter. Signal is indicated with a dotted white circle.

No alteration was observed in MO-control (Figure 38), however, there was an elevated mortality rate (more than 64%) at concentrations above 1.5-2mM (Table 11). In order to avoid MO mistargeting, which leads to developmental defects and high death rates in mammals and fish (Robu, Larson et al. 2007), only the results obtained using 1mM MO were considered.

The microinjection and electroporation directly in limbs at stage 22HH did not promote a successful MO delivered, after ~24 hours it was not possible to observe MO or RFP expression control in the tissues (Figure 40).

Table 11: Percentage of chicken embryos mortality observed after microinjection and electroporation of the test and control experiments for PTHrP and PTH-L MO.

PTHrP		PTHrP*control*		PTH-L		PTH-L*control*	
1mM	*2mM*	*1mM*	*2mM*	*1mM*	*1.5mM*	*1mM*	*1.5mM*
16.6%	64.3%	10.3%	86.6%	9%	63%	11%	66.6%

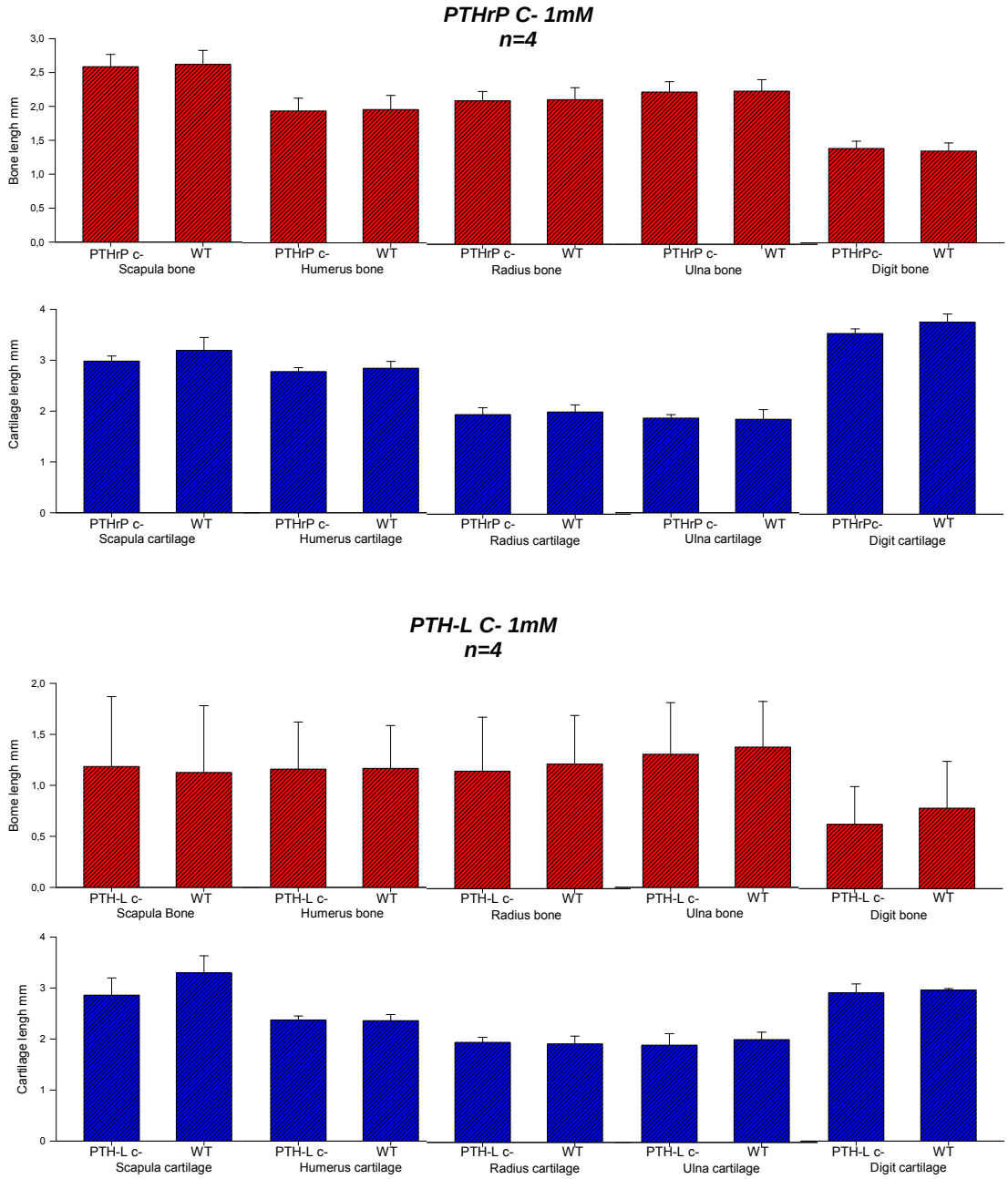


Figure 38: Bar chart of bone and cartilage measurements of chicken embryos 7 days after PTHrP and PTH-L Negative control MO electroporation (n=4). The scapula, humerus, radius, ulna and digit bone/cartilage growth during development were measured. Results are shown as mean ± SEM and C- indicates negative control and WT wild type. No significant differences were observed between treatments and control.

5.3.3. Embryo skeletal measurements

Cartilage and bone from bone/cartilage stained embryos were measured in the different sections, scapula, humerus, radius, ulna and digit 3. However, only in scapula cartilage and in both digit bone and cartilage were observed alterations.

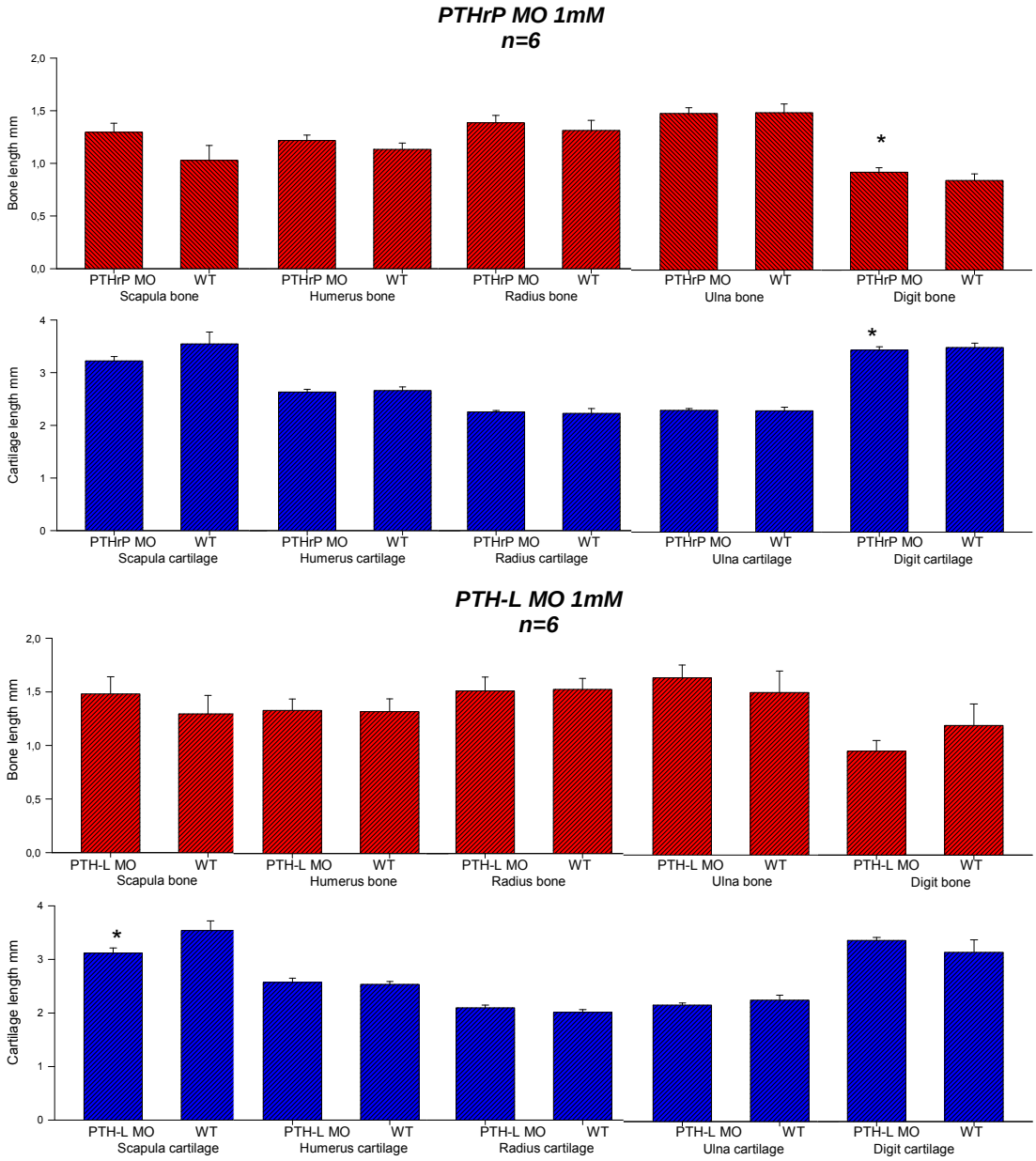


Figure 39: Bar charts of bone and cartilage measurements of chicken embryos 7 days after 1mM PTHrP MO or 1mM PTH-L MO electroporation (n=6). The scapula, humerus, radius, ulna and digit bone/cartilage growth during development were measured. Red represents bone and blue the cartilage. Results are shown as mean ± SEM and the * indicates statistical significance compared to wild type (Walsh, Birch et al.) ($p<0.05$).

In comparison with control wing not electroporated, test electroporations of 1mM PTHrP MO or 1mM PTH-L MO showed a decrease in cartilage length. An increase of bone in digit 3 and a decrease of its cartilage length was observed with 1mM PTHrP MO treatment (Figure 39). However, no significant alterations were observed in other wing

bones or cartilages. 1mM PTH-L MO caused a decrease in scapula cartilage length (Figure 39), however, no alteration in bone length or in other regions was found.

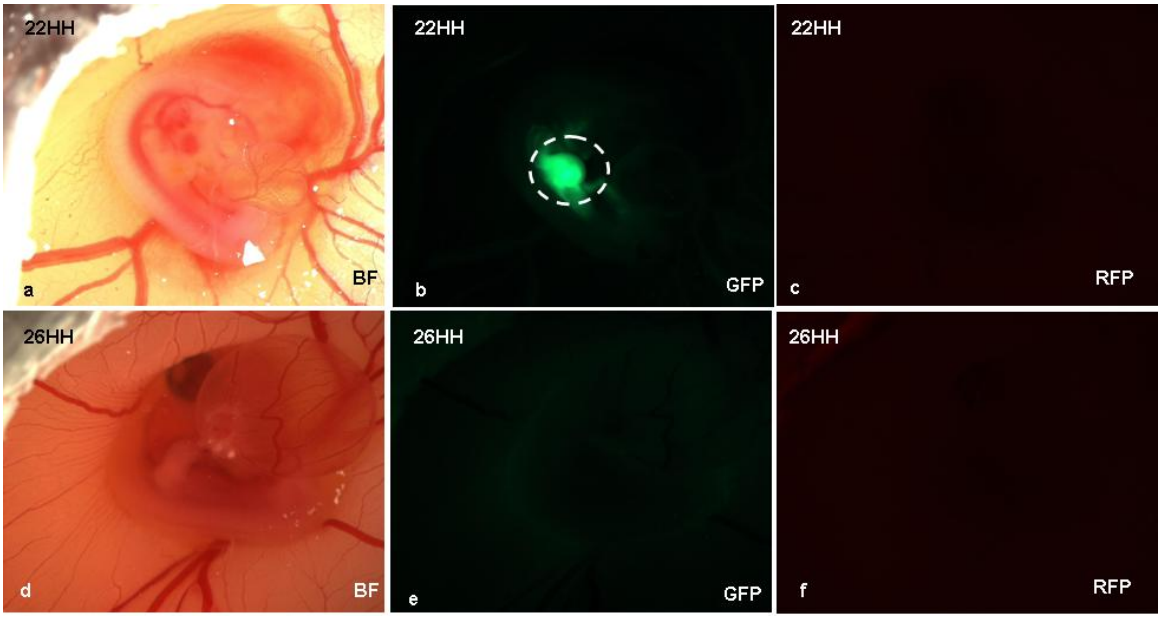


Figure 40: Chicken embryo microinjected and electroporated with MO directly in the right limb. From “a” to “c”, stage 22HH, from “d” to “f”, stage 26HH, with different microscope filters to observe GFP and RFP expression. BF: brighter field, GFP: GFP filter, RFP: texas-red filter. Signal is indicated with dotted white circle

5.4. Discussion

In this study, the role of the PTH system was investigated in chicken embryo development, where an effect of PTHrP and PTH-L in cartilage/bone differentiation was observed.

Despite the failure on the construction of the PTH-family overexpression vectors, which possibly resulted from a technical problem related to RCAS and pCAGGS dephosphorylation or *ClaI* digestion, the success of RCAS and pCAGGS electroporation, with GFP and RFP expression in limbs, suggests a potential technique to use in PTH-family overexpression. Moreover, the observation of expression 7 days after electroporation indicates RCAS as a favourite vector for long time overexpression, since it spreads in the cells and maintains high expression. However, it was not possible to restrict RCAS expression to a specific region.

The results using MO in this study suggest that PTH-family members have a role in skeletal formation during chicken embryo development. As expected, PTHrP knock-down increased bone length suggesting an acceleration of chondrocyte differentiation.

This is in agreement with the observation that deletion of either PTHrP or PTH1R gene leads to acceleration of chondrocyte differentiation and an increase of cortical bone (Lanske, Amling et al. 1999). Moreover, the PTHrP effect on chondrocyte differentiation may reflect its involvement in cartilage and bone morphogenesis, which begins prior to the development of the PTGs and may explain the earlier expression of PTHrP compared to PTH during embryogenesis (chapter IV). PTH-L MO had similar actions to PTHrP on cartilage development, decreasing its length, although with no observed bone alterations. The lack of bone alteration could supports the hypothesis of lost o function of PTH-L along evolution (Pinheiro, Cardoso et al. 2010) and the involvement of unknown receptors interaction, mediating also the apoptosis (Toribio, Brown et al. 2010). The different anatomic region of PTHrP and PTH-L MO actions, affecting only some cartilage and bone regions, could also contributes to the referred hypothesis, however, different delivered efficiencies of MO and the delay of 7 days between electroporation and bone/cartilage staining could mask some structural alterations.

PTHrP was reported to have an anti-apoptotic effect on chondrocytes (Amling, Neff et al. 1997). This process requires the binding to PTH1R, which mediates the protein kinase A (PKA) and protein kinase C (PKC) pathways, being both involved in anti-apoptotic process, however this processes is not yet fully understood (Jilka, Weinstein et al. 1999; Sowa, Kaji et al. 2003; Luparello 2011). The same effect is shared by PTH which instead of increasing osteoblast precursors proliferation, also inhibits osteoblast apoptosis (Jilka, Weinstein et al. 1999) during bone formation (Lynch, Capparelli et al. 1998) and bone fracture healing (Landry, Sadasivan et al. 1997). The comparable action of PTH-L in chondrocytes proliferation suggests a similar pathway through the PTH1R with anti-apoptotic effect as PTH and PTHrP. This hypothesis could explain its expression in cartilage (Pinheiro, Cardoso et al. 2010) and its presence during embryogenesis as early as PTHrP (chapter IV). The different PTH-family expression pattern, differential responses to ambient calcium and their endocrine (PTH) versus paracrine/autocrine (PTHrP) may represent a diversified action during skeleton development, where PTHrP and PTH-L are more likely to play a close, perhaps complementary, role in cartilage development.

Further analysis in chondrocytes cultures using the same approach, should give more clues about the specific role of each of the family members during cartilage differentiation and bone formation. It would be easier to evaluate the effect of knock-down in the cell culture, in view of the fact that the time from microinjection until analysis of results should be smaller in comparison with noticeable effects of MO. In contrast, using whole embryos requires a cavity, such as the coelom or neural tube, to deliver the working solutions (Tucker 2002; Voiculescu, Papanayotou et al. 2008), which in this study compromises the action in bone formation, which initiates only near day 10 (stage 36 HH),

since the used coelom in the wing form region was at stage 13HH. The microinjection and electroporation directly in limbs at stage 22HH, was also tested. However, after ~24 hours it was not possible to observe MO or RFP expression control in the tissues (Figure 40). Due to time restrictions the efficiency of MO in inhibiting mRNA translation was not verified and the results obtained and discussed in this chapter were based only upon empirical observation by comparison of the treated embryos and the control. In the future, successful delivery and elimination of gene expression should be confirmed by immunohistochemistry, to confirm absent or decrease of PTHrP/PTH-L proteins or by the analysis of a gene which expression is dependent of PTHrP/PTH-L.

As conclusion, the PTHrP and PTH-L have a potential role in cartilage and bone development during chicken embryogenesis. The increase of bone length and decrease of cartilage in absent of PTHrP, is in agreement with its anti-apoptotic action. The PTH-L seems to play a similar role in cartilage development, and it was hypothesise a common pathway in this process. Moreover, the previously proposed loss of function of PTH-L during evolution also supports its different actions comparing to PTHrP.

CHAPTER VI

General Discussion

6. General discussion

Calcium is an essential ion obtained from the environment and its endocrine regulation seems to have evolved in accordance to the combination if its availability and requirements. The characterization of the PTH-family members and their receptors, among the most important regulators of calcium homeostasis, is therefore an important aspect to understand the evolution of calcium physiology and its regulatory mechanisms in vertebrates.

6.1. The chicken PTH-system has a conserved role in calcium transport and skeletogenesis

In chicken, as well as *Xenopus*, three genes for PTH-family members (*PTH, PTHrP* and *PTH-L*) are present in the genome and expressed. The observation of their presence also in non-placental mammals highlights the importance of the members of this calciotropic hormone family in relation to the tetrapod radiation and land adaptation, related to the acquisition of a robust bony skeleton structure. PTH-L which in teleosts seems to have similar effectiveness to PTHrP in promoting calcium fluxes (Canario, Rotllant et al. 2006), seems to be a less effective hormone in this role in *Amphibia* and *Aves* (Pinheiro, Cardoso et al. 2010). This may indicate a progressive specialization and in some cases gene loss. Indeed, the gene for *PTH-L* has been lost in eutherian mammals and it appears that these alterations may be possibly linked to the fact that PTH became the main hypercalcemic hormone secreted by an encapsulated gland (parathyroid) that emerged in tetrapods.

The *PTHrP*, which in fishes appears to have an auto/paracrine role on the basis of its wide gene expression, undergoes a much complex regulation in tetrapods, judging from the complexity of gene promoters and transcripts reported in humans and identified in this study for *Xenopus* and chicken. In mammals, *PTHrP* is relatively well characterized, three splice transcripts have been described and a multitude of physiological roles for the protein have been demonstrated (Philbrick, Wysolmersky et al. 1996). This multiple activity may be explained, at least partly, through interaction with multiple receptors, some of which not yet identified (Toribio, Brown et al. 2010).

PTH1R and *PTH3R* were characterised in chicken and shown to have a widespread tissue distribution, largely related to calcium but also during embryo development, suggesting an early role in chicken development and skeletal formation. In human, *PTH1R* and *PTH2R* have been characterised, but *PTH2R*, as well as the neuropeptide *TIP39*, which is its specific ligand, appear to be absent from avian genomes.

Overlapping expression of chicken receptors and peptide transcript in most of tested tissues is suggestive of specific receptor-peptide interactions. *PTHrP* and *PTH1R* were the most widely expressed genes, PTHrP peptide was the most effective in stimulating PTH1R through activation of two independent intracellular signalling pathways. Moreover this peptide is also the preferred ligand in activating PTH3R highlighting the possibility of different physiological mechanisms of action and responses, which is in agreement with its pleotropic role from fish to mammals. The detection of PTHrP and PTH1R in chicken CAM is highly suggestive of their role in regulating calcium availability during embryo development. In contrast, PTH-L demonstrates poor affinity for any of the two receptors and may explain its low calciotropic activity.

During embryogenesis a complex regulatory system involving PTH-family members seems to exist, evidenced by their presence since early stages and with gene specific distribution patterns, where cartilage and bony structures are formed. *PTHrP* expression is detected earlier than *PTH* which further evidences its role has a major regulator of cartilage and bone morphogenesis, starting prior to the development of a PTG. In mammals, PTH and PTHrP are the major hypercalcemic hormones involved in bone turnover. In chicken PTHrP and PTH-L have an important role in skeletogenesis and a clear role for PTH remains to be clarified. During embryogenesis PTHrP directly influences bone length and PTH-L cartilage development. Their role in chicken chondrocytes seems to resemble the anti-apoptotic effect of the mammalian PTHrP, which increases the expression of Bcl-2, a protein that controls programmed cell death, leading to delayed chondrocyte maturation (Amizuka, Warshawsky et al. 1994; Amling, Neff et al. 1997).The anti-apoptotic effect was found to be blocked by a PTH1R antagonist and proposed do be mediated by this receptor (Jilka, Weinstein et al. 1999; Turner, Mefford et al. 2000; Bringhurst 2002). Thus, perhaps in chicken a similar PTH-L pathway through PTH1R is present, in which both PTHrP and PTH-L (however, with less stimulation) activate the PTH1R and share effects in bone/cartilage.

In addition to their action on classical calcium target tissues, the Xenopus and chicken PTH-family members may also have an important role in the vertebrate nervous system. In mammals, PTH has been shown to modulate neuronal calcium levels (Carman, Post et al. 1977; Hull, Fathimani et al. 1998) and PTHrP to act on the differentiation and survival of neuronal cells (Philbrick, Wysolmerski et al. 1996; Hull, Fathimani et al. 1998) with a role as growth/differentiation factor in rat cerebral cortex astrocytes (Struckhoff 1995; Struckhoff and Turzynski 1995; Philbrick, Wysolmerski et al. 1996). In the present study, the PTH-L was also found to be expressed in adult chicken brain and pituitary, *Xenopus* brain and chicken embryo CNS, although its function in this system remains to be studied.

6.2. The PTH-system evolution and calcium regulation

The acquisition of an internal bony skeleton has been related to the emergence of the PTH endocrine system. In fact, both hormones and their corresponding receptors have been isolated and characterised from representatives of distinct vertebrate taxa, including chicken, which is described in this study. Recent studies have also found PTH-family members and PTHRs homologues in cartilaginous fish and invertebrates.

In the elephant shark, three PTH-family genes, two *PTH* (*Pth1* and *Pth2*) and a putative *PTHrP,* were identified and in the urochordate *Ciona intestinalis* homologues of the vertebrate PTHRs are proposed to exist (Cardoso, Pinto et al. 2006; Liu, Ibrahim et al. 2010). Moreover, a series of immunohistochemical studies using human antisera have also detected the presence of PTH-peptide family members in lamprey and sharks and also in snail, cockroach and amphioxus tissues indicating that they are of ancestral origin and emerged prior to protostome-deuterostome divergence (Hull, Fathimani et al. 1998). In invertebrates, bovine PTH was found to stimulate calcium influx in snail neurons and induces depolarization and modulation of neural transmission through the inositol-triphosphate second messenger system (Hull, Fathimani et al. 1998; Hull, Marler et al. 2006). On the basis of these observations it was proposed that calciotropic hormones may have previously acted as neuropeptides before their recruitment to bone development (Hull, Fathimani et al. 1998). However despite availability of molecular data from distinct invertebrate organisms no gene or transcript for a potential PTH-family member has been described. Furthermore, in this study attempts to identify a sequence homologue also failed to retrieve a putative invertebrate gene in publicly available databases.

Based upon available molecular data the evolution of the PTH-system was re-evaluated. PTH-family members and their receptors have arisen early in chordate evolution and seem to have followed the gene or genome duplication events, which are proposed to have influenced vertebrate emergence (Figure 41). Later in evolution a series of selective gene deletions seem to have occurred which may be related with gene functional loss.

Moreover the absence of a homologue of PTH-family peptides in the tunicate genome and the existences of duplicate receptors suggests that PTHRs pre-dated the emergence of their vertebrate ligands. This seems to be a common evolutionary pattern within other vertebrate important endocrine ligand-receptor systems. In fact, similar hypothesis were recently proposed when studying the evolution of the metazoan secretin GPCR members which belong to the same group of receptors than PTHRs (Cardoso, Vieira et al. 2010).

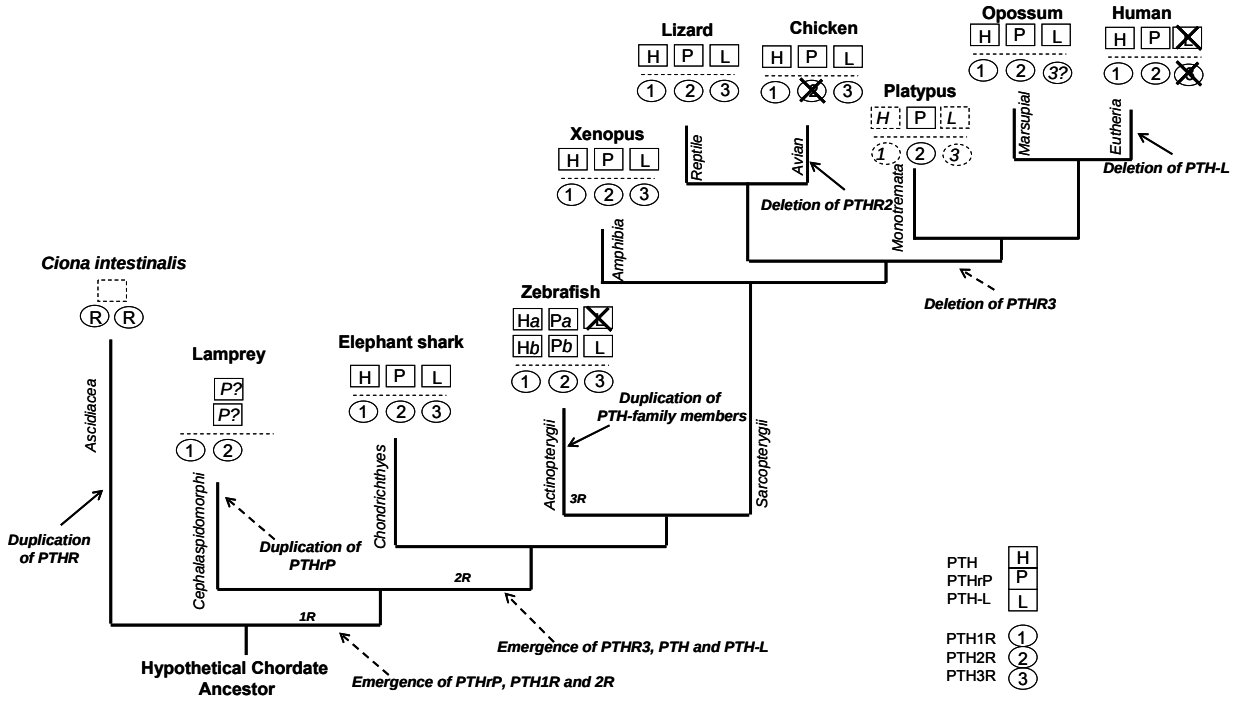


Figure 41: Proposed evolutionary model of the chordate PTH-family members and receptors. Homologues of the human and teleost PTH-family members retrieved from databases are represented by boxes and PTHRs by circles. Dashed arrows indicate potential events in the evolution of the PTH endocrine system and dashed boxes and circles represent genes that were not identified. A cross "X" indicates gene absence and "?" potential gene homology. The proposed rounds of gene/genome duplications that are proposed to influence vertebrate radiation (1R and 2R) and the teleost specific duplication event (3R) are indicated.

Members of the secretin family comprise a small family of brain-gut peptide hormones involved in many physiological functions in vertebrates and are crucial for survival (Sherwood, Krueckl et al. 2000). Orphan expressed secretin family members were also identified in non-vertebrate nematode, insect and *Ciona* genomes, despite failure on the identification of potential ligands. Crosstalk interactions between insect peptides and human secretin members have been demonstrated (Lerner, Iuga et al. 2007) and the reverse scenario also occurs (Cardoso, Coelho et al. 2009). However this has not yet been demonstrated for the metazoan PTH-system. Future studies aiming to functional characterisation of the potential orphan family 2 B1 members in invertebrates, may provide further clues in the understanding of the function and regulation of this system in vertebrates.

Two major rounds of gene/genome duplications, at the emergence of vertebrates (1R), approximately at 500 million years ago (MYA), and a second round (2R) after

jawless fish divergences (approximately 430 MYA), seems to have contributed to fuel organism complexity (Holland, Garcia-Fernandez et al. 1994). Despite the incomplete nature of the analyses of lamprey and elephant shark genomes and the lack of primitive vertebrate genomes available, the hypothetical *PTHR* present in the chordate ancestor has followed this pattern. This revealed by the presence of a *PTH1R* and *PTH2R* homologues in the Agnatha, and of an extra *PTH3R* in the cartilaginous fish genome. In lamprey two incomplete PTHrP-like genes seem to exist and the non-identification of homologues of the vertebrate *PTH* and *PTH-L* may lead to suggest that *PTHrP* was the first PTH-family member to arise. Moreover, a comprehensive and detailed analysis of the elephant shark PTH-family members, including the results obtained in this thesis, revealed that in contrast to what has been previously reported (Liu, Ibrahim et al. 2010) *PTH-L* is also present in cartilaginous genomes along with *PTH* and *PTHrP*. Thus the emergence of the vertebrate *PTH-L* and *PTH* in the genome may have been a consequence of the 2R event. Three receptors and three ligands are maintained in fish, amphibian and reptiles, suggesting that after their emergence conservative gene and functional pressures have contributed for their persistence in the genomes. In teleost fish radiation, a specific duplication event (3R) has occurred by the identification of *PTH* (*PTHa* and *PTHb*) and PTHrP (*PTHrPa* and *PTHrPb*) paralogues (Guerreiro, Renfro et al. 2007). In contrast, a series of selective gene deletion events seem to have occurred during the avian and mammalian divergence. PTH2R was deleted from avian genomes, *PTH3R* is absent from mammals and *PTH-L* is also missing within taxa of the eutheria clade. In Aves, *TIP39* is also absent which in other vertebrates is the preferred ligand of PTH2R and this system plays a role in neuron differentiation in the mammalian hypothalamus (Dobolyi, Palkovits et al. 2010).

The role in the control of calcium homeostasis of PTH-peptides and receptors seem to have emerged early in evolution associated with the acquisition of an internal cartilaginous flexible skeleton (Fuentes, Haond et al. 2007), subsequently different roles were acquired favoured by their evolution in diverse environments. For example in teleosts, corpuscles of Stannius emerged as a specific endocrine gland which secretes stanniocalcin, a hypocalcemic regulator (Wagner and Jaworski 1994; Wagner, Jaworski et al. 1998) facing, in the case of marine fishes, the excess of available free calcium. In these vertebrates, duplicate PTHs are widely distributed, including in nervous system cells (Guerreiro, Renfro et al. 2007) and they seem to play an autocrine or paracrine role in neural function and calcium regulation. It contrasts to the terrestrial environment, where the encapsulated PTG developed firstly in amphibian, and where *PTH* expression is confined largely to this gland (Shapiro and Zwarenstein 1934; Srivastav, Das et al. 1995;

Bergwitz, Klein et al. 1998). This suggests a turning point in the terrestrial colonization in what concerns calcium homeostasis. As food became the principal calcium source and hypercalcemic hormones, produced in a specialized gland, became consequently necessary. PTH became the main hypercalcemic endocrine hormone and PTHrP a paracrine/autocrine/intracrine factor (Philbrick, Wysolmerski et al. 1996; Potts 2005). From its expression pattern PTH-L seems to have acquired a neural function in embryonic development, but either because of redundancy with PTHrP, or some other yet unknown reason, it disappeared from the genome in placental mammals.

The discovery, in chicken, of novel PTH-family members and receptors demonstrating a higher complexity than earlier suspected, constitutes an example of their importance in vertebrate radiation. These findings, together with detailed differential expression and the more complex gene regulation in non-classical calcium target tissues, suggests yet unknown roles. The unique features of chicken in calcium homeostasis and bone development, and the availability of genetic resources make it an excellent model in which to study these questions related to gene function and evolution.

CHAPTER VII

References

7. References

Abbink, W. and G. Flik (2006). "Parathyroid hormone-related protein in teleost fish." General and Comparative Endocrinology **152**(2-3): 243-251.

Alimov, A. P., O. K. Park-Sarge, et al. (2005). "Transactivation of the parathyroid hormone promoter by specificity proteins and the nuclear factor Y complex." Endocrinology **146**(8): 3409-3416.

Altschul, S. F., W. Gish, et al. (1990). "Basic local alignment search tool." J Mol Biol **215**(3): 403-410.

Amizuka, N., H. Warshawsky, et al. (1994). "Parathyroid hormone-related peptide-depleted mice show abnormal epiphyseal cartilage development and altered endochondral bone formation." J Cell Biol **126**(6): 1611-1623.

Amling, M., L. Neff, et al. (1997). "Bcl-2 lies downstream of parathyroid hormone-related peptide in a signaling pathway that regulates chondrocyte maturation during skeletal development." J Cell Biol **136**(1): 205-213.

Ariyan, C. E. and J. A. Sosa (2004). "Assessment and management of patients with abnormal calcium." Crit Care Med **32**(4 Suppl): S146-154.

Armato, U. (2009). "The calcium-sensing receptor: pathophysiology and pharmacological modulation." Curr Pharm Biotechnol **10**(3): 268-269.

Balabanova, S., F. Grosseloh, et al. (1986). "Immunoreactive Parathyroid-Hormone Secretion by Human Placental Tissue." Calcified Tissue International **39**: A103-A103.

Balabanova, S., O. King, et al. (1985). "Distribution and concentration of immunoreactive parathyroid hormone in brain and pituitary of sheep." Klinische Wochenschrift **63**(9): 419-422.

Balabanova, S., J. Peter, et al. (1986). "Parathyroid-Hormone Secretion by the Brain and Pituitary of Sheep." Acta Endocrinologica **111**: 194-196.

Bar, A. (2008). "Calcium homeostasis and vitamin D metabolism and expression in strongly calcifying laying birds." Comp Biochem Physiol A Mol Integr Physiol.

Bar, A. (2009). "Calcium transport in strongly calcifying laying birds: mechanisms and regulation." Comp Biochem Physiol A Mol Integr Physiol **152**(4): 447-469.

Barnicot, N. A. (1948). "The local action of the parathyroid and other tissues on bone in intracerebral grafts." J Anat **82**(Pt 4): 233-248 232.

Bentley, P. J. (1998). Comparative vertebrate endocrinology Melbourne, Cambridge University Press.

Bergwitz, C., P. Klein, et al. (1998). "Identification, functional characterization, and developmental expression of two nonallelic parathyroid hormone (PTH)/PTH-

related peptide receptor isoforms in Xenopus laevis (Daudin)." Endocrinology **139**(2): 723-732.

Bikle, D. (2009). "Nonclassic actions of vitamin D." J Clin Endocrinol Metab **94**(1): 26-34.

Boron, W. F. and E. L. Boulpaep (2009). Medical Physiology: A cellular and Molecular Approach. Philadelphia, Saunders.

Bringhurst, F. R. (2002). "PTH receptors and apoptosis in osteocytes." J Musculoskelet Neuronal Interact **2**(3): 245-251.

Bro, S. and K. Olgaard (1997). "Effects of excess PTH on nonclassical target organs." Am J Kidney Dis **30**(5): 606-620.

Brown, E. M., G. Gamba, et al. (1993). "Cloning and characterization of an extracellular Ca(2+)-sensing receptor from bovine parathyroid." Nature **366**(6455): 575-580.

Brown, W. R., S. J. Hubbard, et al. (2003). "The chicken as a model for large-scale analysis of vertebrate gene function." Nat Rev Genet **4**(2): 87-98.

Campos, R. V., S. L. Asa, et al. (1991). "Immunocytochemical localization of parathyroid hormone-like peptide in the rat fetus." Cancer Res **51**(23 Pt 1): 6351-6357.

Canario, A. V. M., J. Rotllant, et al. (2006). "Novel bioactive parathyroid hormone and related peptides in teleost fish." Febs Letters **580**(1): 291-299.

Cardoso, J., N. Coelho, et al. (2009). Crosstalk between the metazoan family 2 GPCR system. XXXVI International Congress of Physiological Sciences (IUPS2009) Kyoto, Japan.

Cardoso, J. C., V. C. Pinto, et al. (2006). "Evolution of secretin family GPCR members in the metazoa." BMC Evol Biol **6**: 108.

Cardoso, J. C., F. A. Vieira, et al. (2010). "The serendipitous origin of chordate secretin peptide family members." BMC Evol Biol **10**: 135.

Carman, J. S., R. M. Post, et al. (1977). "Calcium and electroconvulsive therapy of severe depressive illness." Biol Psychiatry **12**(1): 5-17.

Chan, S. D., G. J. Strewler, et al. (1990). "Expression of a parathyroid hormone-like protein and its receptor during differentiation of embryonal carcinoma cells." Mol Endocrinol **4**(4): 638-646.

Chiras, D. (1999). Human Biology: Health, Homeostasis, and the Environment. USA, Jones and Bartlett Publishers.

Choi, K. C., P. C. Leung, et al. (2005). "Biology and physiology of Calbindin-D9k in female reproductive tissues: involvement of steroids and endocrine disruptors." Reprod Biol Endocrinol **3**: 66.

Clemens, T. L., S. Cormier, et al. (2001). "Parathyroid hormone-related protein and its receptors: nuclear functions and roles in the renal and cardiovascular systems, the

placental trophoblasts and the pancreatic islets." Br J Pharmacol **134**(6): 1113-1136.

Coccia, P. F., W. Krivit, et al. (1980). "Successful bone-marrow transplantation for infantile malignant osteopetrosis." N Engl J Med **302**(13): 701-708.

Correa, P., G. Akerstrom, et al. (2002). "Underexpression of Gcm2, a master regulatory gene of parathyroid gland development, in adenomas of primary hyperparathyroidism." Clin Endocrinol (Oxf) **57**(4): 501-505.

Cortelyou, J. R. and D. J. McWhinnie (1967). "Parathyroid glands of amphibians. I. Parathyroid structure and function in the amphibian, with emphasis on regulation of mineral ions in body fluids." American Zoologist **7**(4): 843-855.

Cory, S. and J. M. Adams (1988). "Transgenic mice and oncogenesis." Annu Rev Immunol **6**: 25-48.

Cranney, A. and A. Papaioannou (2006). "Parathyroid hormone for the treatment of osteoporosis: a systematic review." CMAJ(175 (1)): 52-59.

Cservenak, M., I. Bodnar, et al. (2010). "Tuberoinfundibular Peptide of 39 Residues Is Activated during Lactation and Participates in the Suckling-Induced Prolactin Release in Rat." Endocrinology **151**(12): 5830-5840.

Cuthbertson, R. M., B. E. Kemp, et al. (1999). "Structure study of osteostatin PTHrP[Thr107](107-139)." Biochim Biophys Acta **1432**(1): 64-72.

Danks, J. A., P. M. Ho, et al. (2003). "Identification of a parathyroid hormone in the fish *Fugu rubripes*." Journal of Bone and Mineral Research **18**(7): 1326-1331.

Danks, J. A., J. C. McHale, et al. (1997). "Parathyroid hormone-related protein in tissues of the emerging frog (*Rana temporaria*): immunohistochemistry and *in situ* hybridization." Journal of Anatomy **190 (Pt 2)**: 229-238.

Davey, M. G. and C. Tickle (2007). "The chicken as a model for embryonic development." Cytogenet Genome Res **117**(1-4): 231-239.

Della Penna, K., F. Kinose, et al. (2003). "Tuberoinfundibular peptide of 39 residues (TIP39): molecular structure and activity for parathyroid hormone 2 receptor." Neuropharmacology **44**(1): 141-153.

Demura, M., T. Yoneda, et al. (2010). "Ectopic production of parathyroid hormone in a patient with sporadic medullary thyroid cancer." Endocr J **57**(2): 161-170.

Diamond, A. G., R. M. Gonterman, et al. (2006). "Parathyroid hormone hormone-related protein and the PTH receptor regulate angiogenesis of the skin." J Invest Dermatol **126**(9): 2127-2134.

Diaz Berenguer, J. A., A. Cabeza Mora, et al. (2002). "[The integrated management software of primary care,a tool for analysing indicators of use]." Aten Primaria **29**(5): 287-293.

Dobolyi, A., M. Palkovits, et al. (2010). "The TIP39-PTH2 receptor system: unique peptidergic cell groups in the brainstem and their interactions with central regulatory mechanisms." Prog Neurobiol **90**(1): 29-59.

Dusso, A. S., A. J. Brown, et al. (2005). "Vitamin D." Am J Physiol Renal Physiol **289**(1): F8-28.

Edwards, H. M., Jr. (2000). "Nutrition and skeletal problems in poultry." Poult Sci **79**(7): 1018-1023.

Etches, R. J. (1987). "Calcium logistics in the laying hen." J Nutr **117**(3): 619-628.

Farquharson, C., D. Jefferies, et al. (2001). "Regulation of chondrocyte terminal differentiation in the postembryonic growth plate: the role of the PTHrP-Indian hedgehog axis." Endocrinology **142**(9): 4131-4140.

Federspiel, M. J. and S. H. Hughes (1997). "Retroviral gene delivery." Methods Cell Biol **52**: 179-214.

Findlay, D. M. and P. M. Sexton (2004). "Calcitonin." Growth Factors **22**(4): 217-224.

Forte, L. R., S. G. Langeluttig, et al. (1982). "Renal parathyroid hormone receptors in the chick: downregulation in secondary hyperparathyroid animal models." Am J Physiol **242**(3): E154-163.

Fox, S. I. (2002). Human Physiology. New York, McGraw-Hill.

Fuentes, J., J. Figueiredo, et al. (2006). "Parathyroid hormone-related protein regulates intestinal calcium transport in the sea bream (*Sparus auratus*)." American Journal of Physiology - Regulatory Integrative and Comparative Physiology **291**(5): R1499-1506.

Fuentes, J., C. Haond, et al. (2007). "Regulation of calcium balance in the sturgeon Acipenser naccarii: a role for PTHrP." Am J Physiol Regul Integr Comp Physiol **293**(2): R884-893.

Fukami, K. (2002). "Structure, regulation, and function of phospholipase C isozymes." J Biochem **131**(3): 293-299.

Garabedian, M., M. F. Holick, et al. (1972). "Control of 25-hydroxycholecalciferol metabolism by parathyroid glands." Proc Natl Acad Sci U S A **69**(7): 1673-1676.

Gardella, T. J. and H. Juppner (2001). "Molecular properties of the PTH/PTHrP receptor." Trends Endocrinol Metab **12**(5): 210-217.

Gardella, T. J., H. Juppner, et al. (1994). "Determinants of [Arg2]PTH-(1-34) binding and signaling in the transmembrane region of the parathyroid hormone receptor." Endocrinology **135**(3): 1186-1194.

Gensure, R. and H. Juppner (2005). "Parathyroid hormone without parathyroid glands." Endocrinology **146**(2): 544-546.

Gensure, R. C., T. J. Gardella, et al. (2005). "Parathyroid hormone and parathyroid hormone-related peptide, and their receptors." Biochemical and Biophysical Research Communications **328**(3): 666-678.

Gensure, R. C., B. Ponugoti, et al. (2004). "Identification and characterization of two parathyroid hormone-like molecules in zebrafish." Endocrinology **145**(4): 1634-1639.

Gether, U. (2000). "Uncovering molecular mechanisms involved in activation of G protein-coupled receptors." Endocr Rev **21**(1): 90-113.

Graves, J. S., B. E. Dunn, et al. (1986). "Embryonic chick allantois: functional isolation and development of sodium transport." American Journal of Physiology **251**(5 Pt 1): C787-794.

Guerreiro, P. M., J. Fuentes, et al. (2001). "Parathyroid hormone-related protein: a calcium regulatory factor in sea bream (Sparus aurata L.) larvae." Am J Physiol Regul Integr Comp Physiol **281**(3): R855-860.

Guerreiro, P. M., J. L. Renfro, et al. (2007). "The parathyroid hormone family of peptides: structure, tissue distribution, regulation, and potential functional roles in calcium and phosphate balance in fish." American Journal of Physiology - Regulatory Integrative and Comparative Physiology **292**(2): R679-696.

Günther, T., Z. F. Chen, et al. (2000). "Genetic ablation of parathyroid glands reveals another source of parathyroid hormone." Nature **406**(6792): 199-203.

Habener, J. F. (1976). "New concepts in the formation, regulation of release, and metabolism of parathyroid hormone." Ciba Found Symp **41**: 197-224.

Habener, J. F., B. W. Kemper, et al. (1976). "Biosynthesis of parathyroid hormone." Recent Progress in Hormone Research **33**: 249-308.

Hamburger, V. and H. L. Hamilton (1992). "A Series of Normal Stages in the Development of the Chick-Embryo, (Reprinted from J Morph, Vol 88, 1951)." Developmental Dynamics **195**(4): 231-272.

Hang, X. M., D. Power, et al. (2005). "Measurement of PTHrP, PTHR1, and CaSR expression levels in tissues of sea bream (Sparus aurata) using quantitative PCR." Ann N Y Acad Sci **1040**: 340-344.

Harmar, A. J. (2001). "Family-B G-protein-coupled receptors." Genome Biol **2**(12): REVIEWS3013.

Harrington, E. K., L. E. Lunsford, et al. (2004). "Chondrocyte terminal differentiation, apoptosis, and type X collagen expression are downregulated by parathyroid hormone." Anatomical Record Part a-Discoveries in Molecular Cellular and Evolutionary Biology **281A**(2): 1286-1295.

Harvey, S., Y. Y. Zeng, et al. (1987). "Parathyroid hormone-like immunoreactivity in fish plasma and tissues." Gen Comp Endocrinol **68**(1): 136-146.

Hatori, M., K. J. Klatte, et al. (1995). "End labeling studies of fragmented DNA in the avian growth plate: evidence of apoptosis in terminally differentiated chondrocytes." J Bone Miner Res **10**(12): 1960-1968.

Hendy, G. N., V. Guarnieri, et al. (2009). "Chapter 3 calcium-sensing receptor and associated diseases." Prog Mol Biol Transl Sci **89**: 31-95.

Hoare, S. R., D. A. Rubin, et al. (2000). "Evaluating the ligand specificity of zebrafish parathyroid hormone (PTH) receptors: comparison of PTH, PTH-related protein, and tuberoinfundibular peptide of 39 residues." Endocrinology **141**(9): 3080-3086.

Hoare, S. R. and T. B. Usdin (2001). "Molecular mechanisms of ligand recognition by parathyroid hormone 1 (PTH1) and PTH2 receptors." Curr Pharm Des **7**(8): 689-713.

Hoenderop, J. G. and R. J. Bindels (2005). "Epithelial Ca2+ and Mg2+ channels in health and disease." J Am Soc Nephrol **16**(1): 15-26.

Hoenderop, J. G., B. Nilius, et al. (2003). "Epithelial calcium channels: from identification to function and regulation." Pflugers Arch **446**(3): 304-308.

Hoenderop, J. G., B. Nilius, et al. (2005). "Calcium absorption across epithelia." Physiol Rev **85**(1): 373-422.

Hogan, B. M., J. A. Danks, et al. (2005). "Duplicate zebrafish pth genes are expressed along the lateral line and in the central nervous system during embryogenesis." Endocrinology **146**(2): 547-551.

Holland, P. W., J. Garcia-Fernandez, et al. (1994). "Gene duplications and the origins of vertebrate development." Dev Suppl: 125-133.

Hong, E. J., K. C. Choi, et al. (2007). "Effects of calcitonin and parathyroid hormone on the regulation of cabindin-D(9k) in the uterus, placenta, and fetal membrane of rats related to blood calcium level during late gestation." Mol Reprod Dev.

Hughes, S. H., J. J. Greenhouse, et al. (1987). "Adaptor plasmids simplify the insertion of foreign DNA into helper-independent retroviral vectors." J Virol **61**(10): 3004-3012.

Hull, K., R. Marler, et al. (2006). "Neural calciotropic peptides: immunoreactive characterization in fish and invertebrates." Neurosci Lett **404**(1-2): 15-19.

Hull, K. L., K. Fathimani, et al. (1998). "Calciotropic peptides: neural perspectives." Comp Biochem Physiol C Pharmacol Toxicol Endocrinol **119**(3): 389-410.

Ieda, T., T. Takahashi, et al. (2000). "Changes in parathyroid hormone-related peptide receptor binding in the shell gland of laying hens (*Gallus domesticus*) during the oviposition cycle." General and Comparative Endocrinology **117**(2): 182-188.

Ingleton, P. and J. Danks (1996). "Distribution and functions of parathyroid hormone-related protein in vertebrate cells." International Review of Cytology **166**: 231-280.

Ingleton, P. M. (2002). "Parathyroid hormone-related protein in lower vertebrates." Comp Biochem Physiol B Biochem Mol Biol **132**(1): 87-95.

Ishibe, M., T. Ishibashi, et al. (1998). "Stimulation of bone formation in vivo by insulin-like growth factor-II in rats." Calcif Tissue Int **63**(1): 36-38.

Ishihara, Y., H. Kamioka, et al. (2008). "Hormonal, pH, and calcium regulation of connexin 43-mediated dye transfer in osteocytes in chick calvaria." J Bone Miner Res **23**(3): 350-360.

Iwata, J., R. Hosokawa, et al. (2010). "Transforming growth factor-beta regulates basal transcriptional regulatory machinery to control cell proliferation and differentiation in cranial neural crest-derived osteoprogenitor cells." J Biol Chem **285**(7): 4975-4982.

Jaillon, O., J.-M. Aury, et al. (2004). "Genome duplication in the teleost fish *Tetraodon nigroviridis* reveals the early vertebrate proto-karyotype." Nature **431**(7011): 946-957.

Jiang, R. S., Z. Xie, et al. (2010). "A single nucleotide polymorphism in the parathyroid hormone gene and effects on eggshell quality in chickens." Poultry Science **89**(10): 2101-2105.

Jilka, R. L., R. S. Weinstein, et al. (1999). "Increased bone formation by prevention of osteoblast apoptosis with parathyroid hormone." J Clin Invest **104**(4): 439-446.

Jin, D. Y., Z. Q. Zhang, et al. (1991). "Evaluation of the purity of recombinant proteins and detection of residual protein contaminants via N-terminal microsequencing and database searching." Chinese Journal of Biotechnology **7**(2): 105-111.

John, M. R., M. Arai, et al. (2002). "Identification and characterization of the murine and human gene encoding the tuberoinfundibular peptide of 39 residues." Endocrinology **143**(3): 1047-1057.

Johnston, M. S. and E. S. Ivey (2002). "Parathyroid and ultimobranchial glands: Calcium metabolism in birds." Seminars in Avian and Exotic Pet Medicine **11**(2): 84-93.

Juppner, H., A. B. Abou-Samra, et al. (1991). "A G protein-linked receptor for parathyroid hormone and parathyroid hormone-related peptide." Science **254**(5034): 1024-1026.

Juppner, H., E. Schipani, et al. (1994). "The extracellular amino-terminal region of the parathyroid hormone (PTH)/PTH-related peptide receptor determines the binding affinity for carboxyl-terminal fragments of PTH-(1-34)." Endocrinology **134**(2): 879-884.

Kamesh, N., G. K. Aradhyam, et al. (2008). "The repertoire of G protein-coupled receptors in the sea squirt *Ciona intestinalis*." Bmc Evolutionary Biology **8**.

Karaplis, A. C., A. Luz, et al. (1994). "Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene." Genes Dev **8**(3): 277-289.

Keutmann, H. T., M. M. Sauer, et al. (1978). "Complete amino acid sequence of human parathyroid hormone." Biochemistry **17**(26): 5723-5729.

Khosla, S. (2001). "Minireview: the OPG/RANKL/RANK system." Endocrinology **142**(12): 5050-5055.

Khosla, S., M. Demay, et al. (1988). "Nucleotide sequence of cloned cDNAs encoding chicken preproparathyroid hormone." Journal of Bone and Mineral Research **3**(6): 689-698.

Kitazawa, S., K. Kajimoto, et al. (2003). "Vitamin D3 supports osteoclastogenesis via functional vitamin D response element of human RANKL gene promoter." J Cell Biochem **89**(4): 771-777.

Klopocki, E., B. P. Hennig, et al. (2010). "Deletion and point mutations of PTHLH cause brachydactyly type E." Am J Hum Genet **86**(3): 434-439.

Koefoed-Johnsen, V. and H. H. Ussing (1958). "The nature of the frog skin potential." Acta Physiol Scand **42**(3-4): 298-308.

Kovacs, C. S., L. L. Chafe, et al. (2001). "PTH regulates fetal blood calcium and skeletal mineralization independently of PTHrP." Endocrinology **142**(11): 4983-4993.

Kovacs, C. S., B. Lanske, et al. (1996). "Parathyroid hormone-related peptide (PTHrP) regulates fetal-placental calcium transport through a receptor distinct from the PTH/PTHrP receptor." Proc Natl Acad Sci U S A **93**(26): 15233-15238.

Krebs, J. and M. Michalak (2007). Clacium: A Matter of Life or Death. Oxford, Elsevier.

Kreth, S., C. Ledderose, et al. (2008). "Differential expression of 5'-UTR splice variants of the adenosine A2A receptor gene in human granulocytes: identification, characterization, and functional impact on activation." FASEB Journal **22**(9): 3276-3286.

Kubota, H., Y. Hayashi, et al. (2005). "Comparison of two methods of in vivo gene transfer by electroporation." Fertil Steril **83 Suppl 1**: 1310-1318.

Kumar, S., K. Tamura, et al. (2004). "MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment." Briefings in Bioinformatics **5**(2): 150-163.

Lacey, D. L., E. Timms, et al. (1998). "Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation." Cell **93**(2): 165-176.

Lagerstrom, M. C., A. R. Hellstrom, et al. (2006). "The G protein-coupled receptor subset of the chicken genome." PLoS Comput Biol **2**(6): e54.

Landry, P., K. Sadasivan, et al. (1997). "Apoptosis is coordinately regulated with osteoblast formation during bone healing." Tissue Cell **29**(4): 413-419.

Lanske, B., M. Amling, et al. (1999). "Ablation of the PTHrP gene or the PTH/PTHrP receptor gene leads to distinct abnormalities in bone development." Journal of Clinical Investigation **104**(4): 399-407.

Lanske, B., A. C. Karaplis, et al. (1996). "PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth." Science **273**(5275): 663-666.

Laverty, G., C. McWilliams, et al. (2003). "PTH stimulates a Cl^--dependent and EIPA-sensitive current in chick proximal tubule cells in culture." American Journal of Physiology-Renal Physiology **284**(5): F987-F995.

Laverty, G., C. McWilliams, et al. (2003). "PTH stimulates a Cl(-)-dependent and EIPA-sensitive current in chick proximal tubule cells in culture." Am J Physiol Renal Physiol **284**(5): F987-995.

Lee, C., M. D. Luck, et al. (1995). "Homolog-scanning mutagenesis of the parathyroid hormone (PTH) receptor reveals PTH-(1-34) binding determinants in the third extracellular loop." Mol Endocrinol **9**(10): 1269-1278.

Lerner, E. A., A. O. Iuga, et al. (2007). "Maxadilan, a PAC1 receptor agonist from sand flies." Peptides **28**(9): 1651-1654.

Li, T. F., Y. Dong, et al. (2004). "Parathyroid hormone-related peptide (PTHrP) inhibits Runx2 expression through the PKA signaling pathway." Exp Cell Res **299**(1): 128-136.

Liu, Y., A. S. Ibrahim, et al. (2010). "Parathyroid hormone gene family in a cartilaginous fish, the elephant shark (Callorhinchus milii)." J Bone Miner Res.

Liu, Z., S. Yu, et al. (2007). "Gcm2 is required for the differentiation and survival of parathyroid precursor cells in the parathyroid/thymus primordia." Dev Biol **305**(1): 333-346.

Logan, M. and C. Tabin (1998). "Targeted gene misexpression in chick limb buds using avian replication-competent retroviruses." Methods **14**(4): 407-420.

Luparello, C. (2011). "Parathyroid Hormone-Related Protein (PTHrP): A Key Regulator of Life/Death Decisions by Tumor Cells with Potential Clinical Applications." Cancers **3**(1): 396-407.

Lynch, M. P., C. Capparelli, et al. (1998). "Apoptosis during bone-like tissue development in vitro." J Cell Biochem **68**(1): 31-49.

Lytton, J. (2007). "Na+/Ca2+ exchangers: three mammalian gene families control Ca2+ transport." Biochem J **406**(3): 365-382.

Mangin, M., K. Ikeda, et al. (1989). "Isolation and characterization of the human parathyroid hormone-like peptide gene." Proc Natl Acad Sci U S A **86**(7): 2408-2412.

Maniatis, T., E. Fritsch, et al. (1982). Molecular Cloning: A Laboratory manual New York.

Mannstadt, M., H. Jüppner, et al. (1999). "Receptors for PTH and PTHrP: their biological importance and functional properties." Am J Physiol **277**(5 Pt 2): F665-675.

Maret, A., I. Bourdeau, et al. (2004). "Expression of GCMB by intrathymic parathyroid hormone-secreting adenomas indicates their parathyroid cell origin." J Clin Endocrinol Metab **89**(1): 8-12.

Martin, T. J. (2005). "Osteoblast-derived PTHrP is a physiological regulator of bone formation." J Clin Invest **115**(9): 2322-2324.

Martyniuk, C. J., K. J. Kroll, et al. (2009). "Seasonal relationship between gonadotropin, growth hormone, and estrogen receptor mRNA expression in the pituitary gland of largemouth bass." General and Comparative Endocrinology **163**(3): 306-317.

McLeod, M. J. (1980). "Differential staining of cartilage and bone in whole mouse fetuses by alcian blue and alizarin red S." Teratology **22**(3): 299-301.

Medill, N. J., C. A. Praul, et al. (2001). "Parathyroid hormone-related peptide expression in the epiphyseal growth plate of the juvenile chicken: evidence for the origin of the parathyroid hormone-related peptide found in the epiphyseal growth plate." Journal of Cellular Biochemistry **80**(4): 504-511.

Mende, M., N. A. Christophorou, et al. (2008). "Specific and effective gene knock-down in early chick embryos using morpholinos but not pRFPRNAi vectors." Mech Dev **125**(11-12): 947-962.

Miao, D., B. He, et al. (2002). "Parathyroid hormone is essential for normal fetal bone formation." J Clin Invest **109**(9): 1173-1182.

Mignone, F., C. Gissi, et al. (2002). "Untranslated regions of mRNAs." Genome Biology **3**(3): reviews0004.

Minina, E., C. Kreschel, et al. (2002). "Interaction of FGF, Ihh/Pthlh, and BMP signaling Integrates chondrocyte proliferation and hypertrophic differentiation." Developmental Cell **3**(3): 439-449.

Mizobuchi, M., C. S. Ritter, et al. (2009). "Calcium-sensing receptor expression is regulated by glial cells missing-2 in human parathyroid cells." J Bone Miner Res **24**(7): 1173-1179.

Moseley, J. M. and M. T. Gillespie (1995). "Parathyroid hormone-related protein." Critical Reviews in Clinical Laboratory Sciences **32**(3): 299-343.

Moseley, J. M., M. Kubota, et al. (1987). "Parathyroid hormone-related protein purified from a human lung cancer cell line." Proc Natl Acad Sci U S A **84**(14): 5048-5052.

Mozo, M. V. and A. Anghel. (2006). "Biology-Online." 2010, from http://www.biology-online.org.

Mueller, W. J., R. Schraer, et al. (1964). "Calcium Metabolism and Skeletal Dynamics of Laying Pullets." J Nutr **84**: 20-26.

Mundy, G. R. and T. A. Guise (1999). "Hormonal control of calcium homeostasis." Clin Chem **45**(8 Pt 2): 1347-1352.

Munson, P. L. (1955). "Studies on the role of the parathyroids in calcium and phosphorus metabolism." Ann N Y Acad Sci **60**(5): 776-796.

Munson, P. L. (1960). "Recent advances in parathyroid hormone research." Fed Proc **19**: 593-601.

Murray, T. M., L. G. Rao, et al. (2005). "Parathyroid hormone secretion and action: evidence for discrete receptors for the carboxyl-terminal region and related biological actions of carboxyl- terminal ligands." Endocrine Reviews **26**(1): 78-113.

Nakamura, H., T. Katahira, et al. (2004). "Gain- and loss-of-function in chick embryos by electroporation." Mech Dev **121**(9): 1137-1143.

Nanjo, K., S. Nagai, et al. (2010). "Identification and functional analysis of novel calcium-sensing receptor gene mutation in familial hypocalciuric hypercalcemia." Endocr J **57**(9): 787-792.

Naveh-Many, T. (2005). Molecular Biology of Parathyroid. New York, Kluwer Academic / Plenum Publisher.

Neer, R. M., C. D. Arnaud, et al. (2001). "Effect of parathyroid hormone (1-34) on fractures and bone mineral density in postmenopausal women with osteoporosis." N Engl J Med **344**(19): 1434-1441.

Nguyen, T. H., G. S. Lee, et al. (2005). "A calcium binding protein, calbindin-D9k, is mainly regulated by estrogen in the pituitary gland of rats during estrous cycle." Brain Res Mol Brain Res **141**(2): 166-173.

Nieto, M. A., K. Patel, et al. (1996). "In situ hybridization analysis of chick embryos in whole mount and tissue sections." Methods Cell Biol **51**: 219-235.

Okabe, M. and A. Graham (2004). "The origin of the parathyroid gland." Proceedings of the National Academy of Sciences of the United States of America **101**(51): 17716-17719.

Orloff, J. J., D. Reddy, et al. (1994). "Parathyroid hormone-related protein as a prohormone: posttranslational processing and receptor interactions." Endocrine Reviews **15**(1): 40-60.

Packard, M. J., N. B. Clark, et al. (1998). "The effect of calcium-regulating hormones on transport of calcium across the chorioallantoic membrane of the chicken embryo."

Comparative Biochemistry and Physiology. Part A, Molecular and Integrative Physiology **119**(2): 547-552.

Panda, D., D. Goltzman, et al. (2009). "TIP39/Parathyroid Hormone Type 2 Receptor Signaling is a Potent Inhibitor of Chondrocyte Proliferation and Differentiation." Am J Physiol Endocrinol Metab.

Panda, D. K., D. Miao, et al. (2004). "Inactivation of the 25-hydroxyvitamin D 1alpha-hydroxylase and vitamin D receptor demonstrates independent and interdependent effects of calcium and vitamin D on skeletal and mineral homeostasis." J Biol Chem **279**(16): 16754-16766.

Pang, P. K., S. Harvey, et al. (1988). "Parathyroid hormone-like immunoreactivity in brains of tetrapod vertebrates." American Journal of Physiology **255**(4 Pt 2): R635-642.

Pang, P. K., T. Kaneko, et al. (1988). "Immunocytochemical distribution of PTH immunoreactivity in vertebrate brains." American Journal of Physiology **255**(4 Pt 2): R643-647.

Papasani, M. R., R. C. Gensure, et al. (2004). "Identification and characterization of the zebrafish and fugu genes encoding tuberoinfundibular peptide 39." Endocrinology **145**(11): 5294-5304.

Pateder, D. B., R. N. Rosier, et al. (2000). "PTHrP expression in chondrocytes, regulation by TGF-beta, and interactions between epiphyseal and growth plate chondrocytes." Exp Cell Res **256**(2): 555-562.

Philbrick, W., J. Wysolmersky, et al. (1996). "Defining the roles of parathyroid hormone-related protein in normal physiology." Physiological Reviews **76**(1): 127-173.

Philbrick, W. M. (1998). "Parathyroid hormone-related protein is a developmental regulatory molecule." Eur J Oral Sci **106 Suppl 1**: 32-37.

Philbrick, W. M., B. E. Dreyer, et al. (1998). "Parathyroid hormone-related protein is required for tooth eruption." Proc Natl Acad Sci U S A **95**(20): 11846-11851.

Pinheiro, P. L., J. C. Cardoso, et al. (2010). "Gene structure, transcripts and calciotropic effects of the PTH family of peptides in Xenopus and chicken." BMC Evol Biol **10**(1): 373.

Pliam, N. B., K. O. Nyiredy, et al. (1982). "Parathyroid hormone receptors in avian bone cells." Proc Natl Acad Sci U S A **79**(6): 2061-2063.

Potts, J. T. (2005). "Parathyroid hormone: past and present." Journal of Endocrinology **187**(3): 311-325.

Potts, J. T. and T. J. Gardella (2007). "Progress, paradox, and potential: parathyroid hormone research over five decades." Ann N Y Acad Sci **1117**: 196-208.

Power, D. M., P. M. Ingleton, et al. (2000). "Genomic structure and expression of parathyroid hormone-related protein gene (PTHrP) in a teleost, Fugu rubripes." Gene **250**(1-2): 67-76.

Rabbani, S. A., J. Mitchell, et al. (1988). "Influence of the amino-terminus on in vitro and in vivo biological activity of synthetic parathyroid hormone-like peptides of malignancy." Endocrinology **123**(6): 2709-2716.

Rhee, Y., S. Y. Park, et al. (2009). "Angiogenesis inhibitor attenuates parathyroid hormone-induced anabolic effect." Biomed Pharmacother **63**(1): 63-68.

Robu, M. E., J. D. Larson, et al. (2007). "p53 activation by knock-down technologies." PLoS Genet **3**(5): e78.

Rodriguez-Commes, J., J. N. Forrest, et al. (1995). "Parathyroid hormone stimulates electrogenic sodium-transport in A6 Cells." Biochemical and Biophysical Research Communications **213**(2): 688-698.

Rosenberg, J., M. Pines, et al. (1989). "Renal and adrenal adenosine 3',5'-monophosphate production and corticosteroid secretion in response to synthetic chicken parathyroid hormone-(1-34)." Endocrinology **125**(2): 1082-1089.

Rotllant, J., P. M. Guerreiro, et al. (2005). "Stimulation of cortisol release by the N terminus of teleost parathyroid hormone-related protein in interrenal cells *in vitro*." Endocrinology **146**(1): 71-76.

Rotllant, J., P. M. Guerreiro, et al. (2006). "Ligand binding and signalling pathways of PTH receptors in sea bream (*Sparus auratus*) enterocytes." Cell and Tissue Research **323**(2): 333-341.

Rotllant, J., B. Redruello, et al. (2005). "Calcium mobilization from fish scales is mediated by parathyroid hormone related protein via the parathyroid hormone type 1 receptor." Regulatory Peptides **132**(1-3): 33-40.

Rubin, D. A., P. Hellman, et al. (1999). "A G protein-coupled receptor from zebrafish is activated by human parathyroid hormone and not by human or teleost parathyroid hormone-related peptide - Implications for the evolutionary conservation of calcium-regulating peptide hormones." Journal of Biological Chemistry **274**(33): 23035-23042.

Rubin, D. A. and H. Juppner (1999). "Zebrafish express the common parathyroid hormone/parathyroid hormone-related peptide receptor (PTH1R) and a novel receptor (PTH3R) that is preferentially activated by mammalian and fugufish parathyroid hormone-related peptide." J Biol Chem **274**(40): 28185-28190.

Russell, J., D. Lettieri, et al. (1983). "Direct regulation by calcium of cytoplasmic messenger ribonucleic acid coding for pre-proparathyroid hormone in isolated bovine parathyroid cells." J Clin Invest **72**(5): 1851-1855.

Russell, J. and L. M. Sherwood (1989). "Nucleotide sequence of the DNA complementary to avian (chicken) preproparathyroid hormone mRNA and the deduced sequence of the hormone precursor." Molecular Endocrinology **3**(2): 325-331.

Safer, J. D., S. Ray, et al. (2007). "A topical parathyroid hormone/parathyroid hormone-related peptide receptor antagonist stimulates hair growth in mice." Endocrinology **148**(3): 1167-1170.

Saitou, N. and M. Nei (1987). "The neighbor-joining method: a new method for reconstructing phylogenetic trees." Molecular Biology and Evolution **4**(4): 406-425.

Schermer, D. T., M. S. Bradley, et al. (1994). "Functional properties of a synthetic chicken parathyroid hormone-related protein 1-36 fragment." Journal of Bone and Mineral Research **9**(7): 1041-1046.

Schermer, D. T., S. D. Chan, et al. (1991). "Chicken parathyroid hormone-related protein and its expression during embryologic development." J Bone Miner Res **6**(2): 149-155.

Schlesinger, N. and R. G. Thiele (2010). "The pathogenesis of bone erosions in gouty arthritis." Ann Rheum Dis **69**(11): 1907-1912.

Schluter, K. D. (1999). "PTH and PTHrP: Similar Structures but Different Functions." News Physiol Sci **14**: 243-249.

Schneider, H., J. H. Feyen, et al. (1993). "Cloning and functional expression of a human parathyroid hormone receptor." Eur J Pharmacol **246**(2): 149-155.

Shapiro, H. A. and H. Zwarenstein (1934). "Metabolic Changes Associated with Endocrine Activity and the Reproductive Cycle in XENOPUS LAEVIS: IV. The Effects of Injection of Ovarian and Pituitary Extracts on the Serum Calcium in Normal, Ovariectomised and Hypophysectomised Toads." J Exp Biol **11**(3): 267-272.

Sharpe, J., U. Ahlgren, et al. (2002). "Optical projection tomography as a tool for 3D microscopy and gene expression studies." Science **296**(5567): 541-545.

Sherwood, N. M., S. L. Krueckl, et al. (2000). "The origin and function of the pituitary adenylate cyclase-activating polypeptide (PACAP)/glucagon superfamily." Endocr Rev **21**(6): 619-670.

Shimizu, M., J. T. Potts, Jr., et al. (2000). "Minimization of Parathyroid Hormone: Novel amino-terminal parathyroid hormone fragments with enhanced potency in activating the type-1 parathyroid hormone receptor." Journal of Biological Chemistry **275**(29): 21836-21843.

Simmonds, C. S., G. Karsenty, et al. (2010). "Parathyroid hormone regulates fetal-placental mineral homeostasis." J Bone Miner Res **25**(3): 594-605.

Sommerfeldt, D. W. and C. T. Rubin (2001). "Biology of bone and how it orchestrates the form and function of the skeleton." Eur Spine J **10 Suppl 2**: S86-95.

Sowa, H., H. Kaji, et al. (2003). "Parathyroid hormone-Smad3 axis exerts anti-apoptotic action and augments anabolic action of transforming growth factor beta in osteoblasts." J Biol Chem **278**(52): 52240-52252.

Srivastav, A. K., V. K. Das, et al. (1995). "Amphibian parathyroids: morphological and functional aspects." Microscopy Research and Technique **32**(2): 79-90.

Srivastav, A. K., V. K. Das, et al. (1995). "Amphibian parathyroids: morphological and functional aspects." Microsc Res Tech **32**(2): 79-90.

Stains, J. P., J. A. Weber, et al. (2002). "Expression of Na(+)/Ca(2+) exchanger isoforms (NCX1 and NCX3) and plasma membrane Ca(2+) ATPase during osteoblast differentiation." J Cell Biochem **84**(3): 625-635.

Starker, L. F., P. Bjorklund, et al. (2010). "Clinical and Histopathological Characteristics of Hyperparathyroidism-induced Hypercalcemic Crisis." World J Surg.

Stiffler, D. F., J. C. Yee, et al. (1998). "Responses of frog skin, Rana pipiens, calcium ion transport to parathyroid hormone, calcitonin, and vitamin D3." General and Comparative Endocrinology **112**(2): 191-199.

Struckhoff, G. (1995). "Transforming growth factor beta 1 and parathyroid hormone-related protein control the secretion of dipeptidyl peptidase II by rat astrocytes." Neurosci Lett **189**(2): 117-120.

Struckhoff, G. and A. Turzynski (1995). "Demonstration of parathyroid hormone-related protein in meninges and its receptor in astrocytes: evidence for a paracrine meningo-astrocytic loop." Brain Res **676**(1): 1-9.

Suda, T., N. Takahashi, et al. (1992). "Role of vitamin D in bone resorption." J Cell Biochem **49**(1): 53-58.

Sugiyama, T. and S. Kusuhara (2001). "Avian calcium metabolism and bone function." Asian-Australasian Journal of Animal Sciences **14**: 82-90.

Sugiyama, T., M. Sakurai, et al. (2004). "Effects of Calcium Regulating Hormones on Osteoclast-Like Cell Formation in Hen Medullary Bone Marrow Culture." J Poult Sci **41**: 307-314.

Suva, L. J. (2006). "PTH expression, not always where you think." J Clin Endocrinol Metab **91**(2): 396-397.

Swarthout, J. T., R. C. D'Alonzo, et al. (2002). "Parathyroid hormone-dependent signaling pathways regulating genes in bone cells." Gene **282**(1-2): 1-17.

Tam, C. S., J. N. Heersche, et al. (1982). "Parathyroid hormone stimulates the bone apposition rate independently of its resorptive action: differential effects of intermittent and continuous administration." Endocrinology **110**(2): 506-512.

Tam, W., S. H. Hughes, et al. (2002). "Avian bic, a gene isolated from a common retroviral site in avian leukosis virus-induced lymphomas that encodes a noncoding RNA,

cooperates with c-myc in lymphomagenesis and erythroleukemogenesis." J Virol **76**(9): 4275-4286.

Teitelbaum, S. L. (2000). "Bone resorption by osteoclasts." Science **289**(5484): 1504-1508.

Teitelbaum, S. L. (2000). "Osteoclasts, integrins, and osteoporosis." J Bone Miner Metab **18**(6): 344-349.

Thiede, M. A. and S. J. Rutledge (1990). "Nucleotide sequence of a parathyroid hormone-related peptide expressed by the 10 day chicken embryo." Nucleic Acids Research **18**(10): 3062.

Thompson, J. D., T. J. Gibson, et al. (1997). "The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools." Nucleic Acids Research **25**(24): 4876-4882.

Tickle, C. (2004). "The contribution of chicken embryology to the understanding of vertebrate limb development." Mech Dev **121**(9): 1019-1029.

Tickle, C. and G. Eichele (1994). "Vertebrate limb development." Annu Rev Cell Biol **10**: 121-152.

Toribio, R. E., H. A. Brown, et al. (2010). "The midregion, nuclear localization sequence, and C terminus of PTHrP regulate skeletal development, hematopoiesis, and survival in mice." FASEB J **24**(6): 1947-1957.

Towers, M. and C. Tickle (2009). "Generation of pattern and form in the developing limb." Int J Dev Biol **53**(5-6): 805-812.

Tucker, R. P. (2002). "Using antisense morpholino oligos to knock-down gene expression in the chicken embryo." Acta Histochemica Et Cytochemica **35**(5): 361-365.

Turner, P. R., S. Mefford, et al. (2000). "Apoptosis mediated by activation of the G protein-coupled receptor for parathyroid hormone (PTH)/PTH-related protein (PTHrP)." Mol Endocrinol **14**(2): 241-254.

Usdin, T. B., C. Gruber, et al. (1995). "Identification and functional expression of a receptor selectively recognizing parathyroid hormone, the PTH2 receptor." J Biol Chem **270**(26): 15455-15458.

Usdin, T. B., S. R. Hoare, et al. (1999). "TIP39: a new neuropeptide and PTH2-receptor agonist from hypothalamus." Nature Neuroscience **2**(11): 941-943.

Venkatachalam, K. and C. Montell (2007). "TRP channels." Annu Rev Biochem **76**: 387-417.

Vitti, D. and E. Kebreab (2010). Phosphorous and Calcium Utilization and Requirements in Farm Animals. London.

Voiculescu, O., C. Papanayotou, et al. (2008). "Spatially and temporally controlled electroporation of early chick embryos." Nat Protoc **3**(3): 419-426.

Wagner, G., E. Jaworski, et al. (1998). "Stanniocalcin in the seawater salmon: structure, function, and regulation." Am J Physiol Regul Integr Comp Physiol **274**(43): R1177-R1185.

Wagner, G. F. and E. Jaworski (1994). "Calcium regulates stanniocalcin mRNA levels in primary cultured rainbow trout corpuscles of stannius." Mol Cell Endocrinol **99**(2): 315-322.

Walsh, C. A., M. A. Birch, et al. (1995). "Expression and secretion of parathyroid hormone-related protein by human bone-derived cells in vitro: effects of glucocorticoids." J Bone Miner Res **10**(1): 17-25.

Webb, A. R. and M. F. Holick (1988). "The role of sunlight in the cutaneous production of vitamin D3." Annu Rev Nutr **8**: 375-399.

Weir, E. C., W. M. Philbrick, et al. (1996). "Targeted overexpression of parathyroid hormone-related peptide in chondrocytes causes chondrodysplasia and delayed endochondral bone formation." Proc Natl Acad Sci U S A **93**(19): 10240-10245.

Whitehead, C. C. (2004). "Overview of bone biology in the egg-laying hen." Poult Sci **83**(2): 193-199.

Whitehead, C. C. and R. H. Fleming (2000). "Osteoporosis in cage layers." Poult Sci **79**(7): 1033-1041.

Wright, E., M. R. Hargrave, et al. (1995). "The Sry-related gene Sox9 is expressed during chondrogenesis in mouse embryos." Nat Genet **9**(1): 15-20.

Wysolmerski, J. J. and A. F. Stewart (1998). "The physiology of parathyroid hormone-related protein: an emerging role as a developmental factor." Annu Rev Physiol **60**: 431-460.

Yamamoto, M., J. G. Seedor, et al. (1995). "Endogenous calcitonin attenuates parathyroid hormone-induced cancellous bone loss in the rat." Endocrinology **136**(2): 788-795.

Zajac, J. D. and J. A. Danks (2008). "The development of the parathyroid gland: from fish to human." Curr Opin Nephrol Hypertens **17**(4): 353-356.

Zhao, Q., P. R. Brauer, et al. (2002). "Expression of parathyroid hormone-related peptide (PTHrP) and its receptor (PTH1R) during the histogenesis of cartilage and bone in the chicken mandibular process." Journal of Anatomy **201**(2): 137-151.

Zuscik, M. J., R. J. O'Keefe, et al. (2002). "Parathyroid hormone-related peptide regulation of chick tibial growth plate chondrocyte maturation requires protein kinase A." Journal of Orthopaedic Research **20**(5): 1079-1090.

Zuscik, M. J., J. E. Puzas, et al. (1994). "Cyclic-AMP-dependent protein kinase activity is not required by parathyroid hormone to stimulate phosphoinositide signaling in chondrocytes but is required to transduce the hormone's proliferative effect." Arch Biochem Biophys **315**(2): 352-361.